RELATIONSHIP OF ABC TRANSPORT PROTEINS WITH HEMATOPOIETIC STEM CELLS AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

The present Application is a Continuation-In-Part of co-pending International Application PCT/US99/11825 filed May 27, 1999, which claims the priority of provisional U.S. Serial No. 60/086,988 filed May 28, 1998, the disclosures of which are hereby incorporated by reference in their entireties. Applicants claim the benefits of these Applications under 35 U.S.C. § § 120 and 119(e).

RESEARCH SUPPORT

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FIELD OF THE INVENTION

The present invention concerns methods of performing *ex vivo* expansion of gene-modified hematopoietic stem cells which are useful for many applications involving bone marrow transplantation and *ex vivo* gene therapy. The present invention also includes the gene-modified hematopoietic stem cells. The present invention further provides a method of isolating stem cells.

BACKGROUND OF THE INVENTION

Hematopoietic stem cells (HSCs) are primitive cells that generate all the formed elements of the blood and immune system. These cells are functionally defined based on their capacity for self-renewal divisions, which leads to the continuous generation of new HSCs over the lifetime of an animal, and by their potential for pluripotent hematopoietic differentiation. There are three possible general outcomes for the

resulting daughter cells when a hematopoietic stem cell divides: (i) differentiation, (ii) self-renewal, or (iii) apoptosis. Despite the extensive study of HSCs, due to its relevance to bone marrow transplantation, gene therapy, and basic hematopoiesis, the mechanisms controlling these three tightly regulated outcomes are poorly understood.

Purification strategies for HSCs have been developed for both mouse [Spangrude *et al.*, *Science* **241**:58-62 (1988):(published erratum appears in *Science* **244**(4908):1030 (1989)); Uchida *et al.*, *J.Exp.Med.* **175**:175-184 (1992)] and humans HSCs [Zanjani *et al.*, *J.Clin.Invest.* **93**:1051-1055 (1994), *see* comments; Larochelle *et al.*, *Nat.Med.* **2**:1329-1337 (1996); Civin *et al.*, *Blood* **88**:4102-4109 (1996)]. Most of these strategies use antibodies directed against various cell surface antigens and multiparameter cell sorting to isolate phenotypically defined cell populations. This approach has allowed isolation of murine stem cell populations of sufficiently high purity to allow reconstitution of irradiated recipients with less than 10 cells [Morrison *et al.*, *Proc.Natl.Acad.Sci.USA* **92**:10302-10306 (1995); Osawa *et al.*, *Science* **273**:242-245 (1996)], while considerably greater numbers of sorted human cells have been required to reconstitute xenogeneic recipients [Larochelle *et al.*, *Nat.Med.* **2**:1329-1337 (1996); Zanjani *et al.*, *Exp.Hematol.* **26**:353-360 (1998), *see* comments].

Hematopoietic stem cells also represent attractive targets for genetic modification since their progeny make up the entire spectrum of the hematopoietic system. Gene therapy involving stem cells is thus an expanding field that potentially has important applications in the treatment of a wide range of diseases [Nienhuis *et al.*, *Cancer*, **67**:2700 (1991)]. However, due to the inherent quiescent nature of stem cells, retroviral gene transfer is limited since stable integration requires cell division. Improved transduction of this target cell population is thus one of the major goals of current gene therapy research. In the mouse, gene transfer and repopulation with genetically-modified bone marrow stem cells following transplantation has been reported [Lemischka *et al.*, *Cell*, **45**:917 (1986) and Dick *et al.*, *Cell*, **42**:71 (1985)]. Whereas the level of stem cell gene transfer and expression are relatively modest, it has been sufficient to investigate effects of gene expression on hematopoiesis [Persons *et al.*, *Blood* ***90***:*1777* (1997)]. In humans, only an extremely low number of transgenic stem cells persist on a long-term basis [Brenner *et al.*, *Lancet*, **342**:1134

(1993) and Brenner, *et al.*, *Lancet*, **341**:85 (1993) and Rill *et al.*, *Blood*, **84**:380 (1994)]. Therefore there is a need for increasing the proportion of such transduced stem cells through *ex vivo* expansion following transduction and/or through *in vivo* selection approaches.

Most current protocols for transduction of stem cells employ *in vitro* liquid suspension culture with hematopoietic growth factors. It is now well established that culturing murine bone marrow cells for 4 days in the presence of defined concentrations of interleukin-3, interleukin-6, and stem cell factor does not adversely effect overall stem cell survival and function. However, expansion beyond this point has not proven to be beneficial and results in depletion of the reconstitution potential of the bone marrow graft. Cytokine-stimulated stem cells cultured in expansion conditions typically either undergo differentiation or programmed cell death (apoptosis). More mature populations such as the CFU-S and CFU-C, however, are capable of significant expansion in culture. However, these cells are distinct from stem cells and only provide short to moderate-term repopulating ability in transplanted mice. In humans, the long-term culture-initiating cell (LTC-IC) can be expanded *in vitro* with appropriate combinations and amounts of growth factors. LTC-ICs have recently been shown to be more mature than the SCID mouse repopulating cell (SRC) [Dick *et al.*, *Cell* **42**:71 (1985)]. SRCs are depleted in cultures that are more than 4 days old, which is consistent with the SRC being a more primitive cell type. More recently, culturing hematopoietic stem cells derived from the AGM (a pre-liver intraembryonic site) has been reported [Dzierzak *et al.* WO 98/12304, hereby incorporated by reference in its entirety]. However, the prior art teaches at most a four-fold expansion of human hematopoietic cells [Bhatia *et al.*, J. Exp. Med., **186**:619-624 (1997)].

The human MDR1 gene and its murine homologs were originally identified based on the ability of their expressed products, collectively referred to as P-glycoproteins (P-gps), to extrude a wide variety of cytotoxic drugs from the cell interior [Gros *et al.*, *Cell*, **47**:371-380 (1986) and Chen *et al.*, *Cell*, **47**:381-389 (1986)]. It is now known that the MDR1 gene belongs to a superfamily of transport proteins that contain a conserved ATP-binding cassette (ABC) which is necessary for pump function

[Allikmets *et al.*, *Hum. Mol. Genet.* **5**:1649-1655 (1996)]. Numerous studies have clearly shown that P-gp expression plays an important role in the resistance of human tumor cells to cancer chemotherapy [Pastan and Gottesman, *Annu. Rev. Med.*, **42**:277-286 (1991)]. Considering that P-gps are also expressed in a wide variety of normal tissues, more recent studies have examined the normal physiologic functions of MDR1-like genes. Murine gene disruption experiments have demonstrated that expression of various P-gps is necessary for biliary excretion [Smit *et al.*, *Cell*, **75**:451-462 (1993)], maintenance of the blood-brain barrier [Schinkel *et al.*, *Cell*, **77**:491-502 (1994)], and elimination of drugs [Schinkel *et al.*, *Proc.Natl.Acad.Sci. USA*, **94**:4028-4033 (1997)]. P-gps can also mediate more general cellular functions including the translocation of lipids across the cell membrane [van Helvoort *et al.*, *Cell*, **87**:507-517 (1996)] and modulation of specific apoptosis pathways [Johnstone *et al.*, *Blood*, **93**:1075-1085 (1999) and Smyth *et al.*, *Proc.Natl.Acad.Sci.USA*, **95**:7024-7029 (1998)].

P-gp is expressed in a variety of hematopoietic cell types [Drach *et al.*, *Blood*, **80**:2729-2734 (1992)], including human CD34+ stem cells [Chaudhary and Roninson, *Cell*, **66**:85-94 (1991)] and murine c-kit+ stem cells [Sorrentino *et al.*, *Blood*, **86**:491-501 (1995)]. Several lines of evidence suggest that P-gp expression is functionally conserved in hematopoietic stem cells.

Another ATP transport protein that contains a conserved ATP-binding cassette is the gene product of the *Bcrp1/Mxr/Abcp* gene (referred to as *Bcrp* and BCRP hereafter). The BCRP cDNA was originally cloned from several different human tumor cell lines that were resistant to multiple drugs including doxorubicin, topotecan, and mitoxantrone [Doyle *et al.*, *Proc.Natl.Acad.Sci.USA* **95**:15665-15670 (1998):(published erratum appears in *Proc Natl Acad Sci U S A*; **96**(5):2569 (1999)); Maliepaard *et al.*, *Cancer Res.* **59**:4559-4563 (1999); Miyake *et al.*, *Cancer Res.* **59**:8-13 (1999)]. A highly related mouse homologue (*Bcrp1*) was cloned from fibroblasts selected for multidrug resistance [Allen *et al.*, *Cancer Res.* 59:4237-4241 (1999)]. In contrast to the structure of the MDR1 gene, which consists of two duplicated halves, the predicted structure of BCRP is that of a "half transporter", with a single ATP binding cassette and transmembrane region. The expression pattern of

BCRP is highly restricted in normal human tissues, with the highest levels of mRNA detected in the placenta, and much lower levels detected in adult organs [Doyle *et al.*, *Proc.Natl.Acad.Sci.USA* **95**:15665-15670 (1998):(published erratum appears in *Proc.Natl.Acad.SciU S A.* **96**(5):2569 (1999)); Allikmets *et al.*, *CancerRes.* **58**:5337-5339 (1998)].

Hematopoietic stem cells can be identified based on their ability to efflux fluorescent dyes that are substrates for P-gp, such as Rhodamine (Rho) 123 [Spangrude and Johnson, *Proc.Natl.Acad.Sci. SA*, **87:**7433-7437 (1990); Fleming *et al.*, *J. Cell Biol.*, **122**:897-902 (1993); Orlic *et al.*, *Blood*, **82**:762-770 (1993); and Zijlmans *et al.*, *Proc.Natl.Acad.Sci.USA*, **92**:8901-8905 (1995)] and Hoechst 33342 [McAlister *et al.*, *Blood*, **75**:1240-1246 (1990); Wolf *et al.*, *Exp. Hematol.*, **21**:614-622 (1993); and Leemhuis *et al.*, *Exp. Hematol.*, **24**:1215-1224 (1996)]. One particular approach for purifying stem cells is based on Hoechst dye-staining of bone marrow cells to identify a minor fraction of side population (SP) cells that are highly enriched for repopulating activity [Goodell *et al.*, *J. Exp. Med.*, **183**:1797-1806 (1996)]. This SP phenotype identifies a primitive subset of stem cells present in multiple mammalian species [Goodell *et al.*, *Nat. Med.*, **3**:1337-1345 (1997)], and based on verapamil inhibition studies, may be due to expression of P-gp or another ABC transporter [Goodell *et al.*, *J. Exp. Med.*, **183**:1797-1806 (1996)].

Methodology for enriching pluripotent stem cells in culture could have a major impact on treatment of blood and immune-system disorders. For example, bone marrow transplantation is often the only option for persons having hematopoietic and immune-system dysfunctions caused by congenital disorders and/or chemotherapy or radiation therapy. In addition, enriching pluripotent stem cells should greatly enhance the treatment of immunodeficency disorders. Furthermore, the effectiveness of the treatment of blood diseases by *ex vivo* gene therapy, *e.g.*, treating sickle cell anemia or thalassemia, could also be substantially enhanced. Therefore, expansion of primitive stem cells in culture would be a major advance for all aspects of bone marrow transplantation as well as gene therapy applications. Unfortunately, despite the clear need for such methodology, heretofore, it has not been realized.

In addition, whereas a recent report demonstrates that sorting for expression of the vascular endothelial growth factor receptor can enrich human stem cells to near purity [Ziegler *et al.*, *Science* **285**:1553-1558 (1999)], there still remains a general need for better and more specific markers of human HSCs.

The citation of any reference herein should not be deemed as an admission that such reference is available as prior art to the instant invention.

SUMMARY OF THE INVENTION

The present invention provides a method of performing *ex vivo* expansion of a gene-modified hematopoietic stem cell. One embodiment of this type comprises transducing a hematopoietic stem cell with a nucleic acid encoding a transmembrane efflux pump (an ABC transporter) and then culturing the transduced cell, *i.e.*, the gene-modified hematopoietic stem cell, *ex vivo*, thereby expanding the gene-modified hematopoietic stem cell. In a preferred embodiment, the gene-modified hematopoietic stem cell is expanded at least 10-fold.

As exemplified below, in one embodiment the transmembrane efflux pump can be human multidrug resistance-1 (*i.e.*, MDR1, the P-glycoprotein) which is encoded by the nucleotide sequence of SEQ ID NO:1 and has the amino acid sequence of SEQ ID NO:2 . In another embodiment the transmembrane efflux pump is murine MDR1 which is encoded by the nucleotide sequence of SEQ ID NO:5 and has the amino acid sequence of SEQ ID NO:6. In yet another embodiment the transmembrane efflux pump is murine MDR-3 which is encoded by the nucleotide sequence of SEQ ID NO:7 and has an amino acid sequence of SEQ ID NO:8. In still another embodiment the transmembrane efflux pump is the human *Bcrp1/Mxr/Abcp* gene product (BCRP) which is encoded by the nucleotide sequence of SEQ ID NO:9 and has the amino acid sequence of SEQ ID NO:10. In yet another embodiment the transmembrane efflux pump is a murine BCRP which is encoded by the nucleotide sequence of SEQ ID NO: 13 and has the amino acid sequence of SEQ ID NO: 14. In still another embodiment the transmembrane efflux pump is a murine BCRP which is encoded by a nucleotide

sequence comprising SEQ ID NO:11 and has an amino acid sequence comprising SEQ ID NO:12.

In a particular embodiment inhibitors of P-glycoprotein, such as PSC833, are added to the expansion culture. In yet another embodiment the transmembrane efflux pump is MRP (multidrug resistant protein). In still another embodiment, the transmembrane efflux pump is the cystic fibrosis membrane transporter.

In one embodiment, the method comprises culturing the gene-modified hematopoietic cell in the presence of one or more cytokines. In one such embodiment the culture contains 5 to 300 ng/ml of the cytokine. In a particular embodiment the culture contains 10 to 50 ng/ml of the cytokine. In another embodiment the culture contains 0.5 to 10 ng/ml of the cytokine.

In one embodiment the cytokine is an early-acting hematopoietic cytokine. In a particular embodiment the cytokine is interleukin-3. In another embodiment the cytokine is interleukin-6. In still another embodiment the cytokine is stem cell factor. In still another embodiment the cytokine is G-CSF. In yet another embodiment the cytokine is GM-CSF. In still another embodiment the cytokine is the FLT-3 ligand. In yet another embodiment the cytokine is interleukin-1. In still another embodiment more than one of these cytokines are present. In a particular embodiment interleukin-3, interleukin-6, and stem cell factor are all present.

In another embodiment of the method of performing *ex vivo* expansion of a gene-modified hematopoietic stem cell, the cell is expanded for at least 3 days. In alternative embodiment the gene-modified hematopoietic stem cell is expanded for at least 6 days. In a particular embodiment the gene-modified hematopoietic stem cell is expanded for at least 9 days. In a preferred embodiment the gene-modified hematopoietic stem cell is expanded for at least 12 days. In a related embodiment the gene-modified hematopoietic stem cell further comprises a second heterologous gene.

In still another embodiment of the method of performing *ex vivo* expansion of a gene-modified hematopoietic stem cell, the hematopoietic stem cell is a mammalian

hematopoietic stem cell. In a particular embodiment the mammalian hematopoietic stem cell is a murine hematopoietic stem cell. In a preferred embodiment the mammalian hematopoietic stem cell is a human hematopoietic stem cell. In another preferred embodiment the gene-modified hematopoietic stem cell expresses a splice-corrected version of the human MDR1, as exemplified below.

A particular method of the present invention comprises transducing the hematopoietic stem cell with a viral vector that comprises a nucleic acid encoding a transmembrane efflux pump. In one such embodiment the transmembrane efflux pump is MDR1. In another embodiment the transmembrane efflux pump is BCRP. In a particular embodiment the viral vector is a herpes simplex viral vector. In another embodiment the viral vector is an adenoviral vector. In still another embodiment the viral vector is an adeno-associated viral vector (AAV). In a preferred embodiment the viral vector is a defective virus, more preferably not encoding a gene for a functional viral protein.

In an alternative embodiment of the method, the viral vector is a retroviral vector. In one such embodiment the retroviral vector is an HIV retroviral vector. In another embodiment the vector is a VL 30 vector. In yet another embodiment the vector is a MSCV retroviral vector. As exemplified below the retroviral vector can be a Harvey Murine Sarcoma Vector. In one such embodiment the hematopoietic stem cell is transduced by being co-cultured with a retroviral producer cell line. In still another embodiment of the method, transducing the hematopoietic stem cell with a transmembrane efflux pump, *e.g.*, MDR1or BCRP, is performed with a DNA vector (*i.e.*, a naked DNA) that comprises a nucleic acid encoding the transmembrane efflux pump.

In a particular embodiment the nucleic acid encoding a transmembrane efflux pump, *e.g.*, MDR1 or BCRP, is introduced into the hematopoietic stem cell with a non-integrating vector *e.g.*, an adenoviral vector. Such an adenoviral vector would only be expressed transiently, during the period of *in vitro* expansion. This contrasts with the retroviral vector exemplified below which is integrated and expressed continuously *in vivo*. In another embodiment, the nucleic acid encoding MDR1 or BCRP, for example, is introduced into the hematopoietic stem cell with Murine Stem Cell Virus

which lacks the VL30 sequences in the Harvey Murine Sarcoma vector [Hawky *et al.*, *Gene Therapy* 1:136 (1994)].

The present invention further provides a gene-modified hematopoietic stem cell that has been transduced with a nucleic acid encoding a transmembrane efflux pump, *e.g.* MDR1 or BCRP, and has been expanded. In one embodiment the hematopoietic stem cell is a mammalian hematopoietic stem cell. In a particular embodiment the hematopoietic stem cell is a murine hematopoietic stem cell. In a preferred embodiment the mammalian hematopoietic stem cell is a human hematopoietic stem cell. In another preferred embodiment the gene-modified hematopoietic stem cell expresses a splice-corrected version of the human MDR1 as exemplified below.

In one embodiment the gene-modified hematopoietic stem cell has been expanded for at least 3 days. In another embodiment the gene-modified hematopoietic stem cell has been expanded for at least 6 days. In a particular embodiment the gene-modified hematopoietic stem cell has been expanded for at least 9 days. In a preferred embodiment the gene-modified hematopoietic stem cell of has been expanded for at least 12 days. In a particular embodiment of this type the gene-modified hematopoietic stem cell has been expanded for 16 days or more. In a related embodiment the gene-modified hematopoietic stem cell further comprises a second heterologous gene.

Methods of engrafting an animal with the gene-modified hematopoietic stem cell of the present invention are also provided. Preferably, the gene-modified hematopoietic stem cell has been expanded as taught herein. One embodiment comprises placing the expanded gene-modified hematopoietic stem cell into an animal. In one such method, placing the cell into the animal is performed by injection. In a particular embodiment more than one injection is made. In another embodiment multiple injections are made over the course of several days (*e.g.*, in humans 1 to 20 days appears to be a reasonable range). In one embodiment the animal is a mammal. In a particular embodiment the mammal is a mouse. In a preferred embodiment the mammal is a human. Preferably the engrafted cell is stable for at least three months, and more preferably six months, or a year or even longer.

The present invention further provides methods of treating an animal in need of treatment for a hematopoietic stem cell deficiency using a method of engrafting of the present invention. In one embodiment of this method the hematopoietic stem cell is transduced *ex vivo* with a nucleic acid encoding a transmembrane efflux pump, *e.g.* MDR1 or BCRP. The transduced hematopoietic stem cell (a gene-modified hematopoietic stem cell) is expanded and then engrafted into the animal. In a preferred embodiment, the hematopoietic stem cell is obtained from the animal in need of treatment, and then after being transduced with a nucleic acid encoding MDR1 or BCRP and expanded, the resulting gene-modified hematopoietic stem cell is placed back into the animal. In a particular embodiment the animal is a mammal. In a preferred embodiment of this type, the mammal is a human.

A gene-modified hematopoietic stem cell used in a method of engrafting an animal of the present invention can further comprise a second heterologous gene. Such methods include *ex vivo* gene therapy which may be used to treat diseases involving a dysfunctional cell that is derived from an hematopoietic stem cell. Thus, any genetic defect that could be corrected by bone marrow transplantation can be treated by the methods described herein. In one such embodiment, the second heterologous gene encodes a functional β-globin. In another embodiment, the second heterologous gene encodes a functional adenosine deaminase. In still another embodiment, the second heterologous gene encodes a functional glucocerebrosidase.

The present invention further provides the use of the expression of a transmembrane efflux pump, as a means to purify stem cells. Indeed, the present invention discloses that BCRP expression is a specific marker for stem cells *e.g.*, hematopoietic stem cells, and side population (SP) stem cells from other organs. The present invention therefore provides methods for isolating primitive stem cells based on the detection of BCRP expression, which as disclosed herein, is a functional determinant for the SP cell phenotype.

Therefore, the present invention provides methods of identifying stem cells. One such embodiment comprises obtaining a cell sample which contains (or is suspected to contain) stem cells and detecting the expression of BCRP in the cell sample. A cell is

is identified as a stem cell if BCRP is expressed by the cell. The detection of the expression of BCRP can be performed *via* its specific pumping activity. Preferably the detecting of the expression of BCRP is performed with an anti-BCRP antibody which binds to BCRP (more preferably the extracellular portion of BCRP). Stem cells are identified due to their binding to the anti-BCRP antibody. The cell sample can be obtained from any animal, but preferably a mammal and more preferably a human.

The identification and isolation of stem cells *via* the methods of the present invention extend beyond hematopoietic stem cells and comprises all stem cells, including muscle stem cells, and even brain stem cells. The present invention also provides methods of using these isolated stem cells including the use of muscle stem cells in the treatment of diseases such as muscular dystrophy, and Parkinson's Disease. In addition, the hematopoietic stem cells can be used in bone marrow transplants (*e.g.*, for treatment of leukemia) as well as for *ex vivo* gene therapy for treatment of blood diseases such as sickle cell anemia and thalassemia.

The present invention therefore also provides methods of isolating stem cells. One such embodiment comprises obtaining a cell sample which contains (or is suspected to contain) stem cells and contacting them with an antibody that binds to BCRP (preferably an extracellular portion BCRP). Cells that bind to the antibody are then isolated. These isolated cells are identified as isolated stem cell due to their binding to the anti-BCRP antibody. The cell sample can be obtained from any animal, but preferably a mammal and more preferably a human. In a preferred embodiment of the present invention the isolation of the stem cells is performed by flow cytometry. In particular embodiment, the antibody has a fluorescent label and the isolation of the stem cells is performed by fluorescent-activated cell sorting (FACS).

In another embodiment, the anti-BCRP antibody is placed on a solid support. The solid support can then be contacted/incubated with a sample of cells, such that the cells can associate with the solid support by binding to the anti-BCRP antibody. The solid support is then washed to remove cells that bind non-specifically. The remaining cells are eluted from the solid support (by an excess of free antibody, for

example). Based on their ability to bind anti-BCRP antibody with specificity, the eluted cells are identified as isolated stem cells. In a particular embodiment, the solid support is an immunomagnetic bead (*e.g.*, MILTENYI MINIMACS™, DYNABEADS™). The anti-BCRP antibody is placed on the immunomagnetic beads which are then contacted/incubated with a sample of cells, as indicated above, such that the cells can associate with the beads by binding to the anti-BCRP antibody. Preferably after an appropriate incubation period, the immunomagnetic beads can then be separated from the sample of cells with a magnet. The immunomagnetic beads are then washed to remove cells that bind non-specifically. The remaining cells are eluted from the immunomagnetic beads as indicated above. Again, based on their ability to bind anti-BCRP antibody, the isolated cells are identified as stem cells.

The present invention also provides a method of diagnosing and/or prognosing human acute myelogenous leukemia (AML) through assaying BCRP expression in leukemic cells, *e.g.*, blast cells from individuals having or suspected of having AML. Such diagnosis can be used to tailor a therapeutic regimen so as to contain drugs that are not susceptible to the counter-effects of the BCRP (such as being pumped out of the cell by this transmembrane efflux pump). In addition, the detection of the expression of BCRP in cells from a bone marrow sample or a blood sample can lead to the early diagnosis and/or prognosis of AML. Such early diagnosis and/or prognosis could lead to particular treatments such as an earlier bone marrow transplantation. The detection of BCRP expression in the cells can be performed with an anti-BCRP antibody using flow cytometry and/or immunocytochemistry.

Therefore, the present invention provides methods of diagnosing AML in a human subject. A particular embodiment of this type comprises obtaining a leukemic cell from the subject and then determining whether BCRP is overexpressed in the leukemic cell. When BCRP is determined to be overexpressed in the leukemic cell, the patient is diagnosed as having AML. In a preferred embodiment the leukemic cell is a blast cell.

Accordingly, it is a principal object of the present invention to provide a method of expanding hematopoietic stem cells *ex vivo*.

It is a further object of the present invention to provide an expanded hematopoietic stem cell.

It is a further object of the present invention to provide a method for reconstituting bone marrow cells in an animal subject after the animal has undergone chemotherapy or radiation therapy.

It is a further object of the present invention to provide a method for providing bone marrow cells for a human subject while the human is undergoing chemotherapy.

It is a further object of the present invention to provide a method of engrafting a gene-modified hematopoietic stem cell into an animal subject.

It is a further object of the present invention to provide a method of performing *ex vivo* gene therapy on an animal subject by engrafting an expanded gene-modified hematopoietic stem cell into the animal subject wherein the gene-modified hematopoietic stem cell further comprises a therapeutic gene.

It is a further object of the present invention to provide a method of obtaining purified stem cells.

It is a further object of the present invention to provide the purified stem cells.

It is a further object of the present invention to provide methods of using the purified stem cells in the treatment of diseases in which one or more specific cell types are being adversely depleted and/or become dysfunctional.

It is a further object of the present invention to provide methods of using the purified stem cells for gene therapy.

It is a further object of the present invention to use BCRP as a marker for diagnosing dysfunctional cells in humans.

It is a further object of the present invention to use BCRP as a marker for prognosing the progression of AML.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1B show the expansion kinetics for total cells (Figure 1A) and drug-resistant progenitors (Figure 1B) following retroviral transduction. Cells were maintained in liquid suspension cultures with addition of murine IL-3, human IL-6, and rat SCF. Figure 1A shows a typical cell expansion for cells from either MDR1 or DHFR co-cultures. No significant difference in cell expansion was noted between these groups. Cells were removed at 6 day intervals and assayed for clonogenic progenitors in methylcellulose. Selective concentrations of taxol or trimetrexate were used to determine MDR1 or DHFR expressing progenitor cells respectively. Figure 1B shows the drug-resistant progenitor population was found to expand extensively and typically reached 100-fold by 2 weeks.

Figures 2A-2B show the long-term analysis of engraftment with donor bone marrow in non-irradiated recipients. HW80 recipient mice were injected for 5 consecutive days with transduced bone marrow cells (C57) which had been expanded in culture for 12 to 16 days. Later the same day, mice were treated with trimetrexate (130 mg/kg) and NBMPR-P (20 mg/kg). Beginning at 1 week post-transplant, donor C57 hemoglobin levels were quantitated by electrophoresis on cellulose acetate gels. Persistent engraftment was only seen in mice receiving expanded bone marrow cells transduced with HaMDR1 (5/12) as shown in Figure 2B. Engrafted mice included: MDR 7 (■), MDR 11 (▲), MDR 18 (◆), MDR 20 (▼) and from the second experiment MDR 15 (●). No stable engraftment was seen in mock-transduced (0/8) or DHFR-transduced (0/8) expanded bone marrow as shown in Figure 2A. Shown in Figures 2A-2B are mice from 2 independent expansion experiments.

Figures 3A-3C show representative hemoglobin electrophoresis gels from non-irradiated mice engrafted with expanded bone marrow (5 months post-BMT for MDR 20 and 7 months post-BMT for all others). C57 bone marrow was used as the donor marrow for transduction and expansion. Recipient mice were HW80. The differing hemoglobin patterns are indicated in Figure 3A. Primary recipients shown in Figure 3B are MDR 7, 11, 18, 20 from expt. #1 and MDR 15 from expt. #2. Secondary irradiated recipients were transplanted with marrow cells from MDR 20 demonstrating persistence of donor engraftment following secondary transplant, (a formal proof of stem cell engraftment) as shown in Figure 3C. Figure 3D shows the FACS analysis for P-glycoprotein (Pgp) expression in red blood cells from engrafted mice. Peripheral red blood cells were stained with a monoclonal antibody to human Pgp followed by FACS analysis for the PE chromophore. As a negative control, a mouse injected with HaDHFR-transduced marrow (DHFR #1) is shown. All 4 mice from expt. #2 analyzed at 10 weeks post-transplant demonstrate significant levels of Pgp positive red blood cells. When corrected for the percent donor red cells present at the time of analysis, levels of Pgp positivity approached 100% of circulating donor red blood cells.

Figures 4A-4C show the competitive repopulation assay to determine the relative stem cell content of *ex vivo* expanded bone marrow versus fresh non-expanded marrow. C57 donor bone marrow cells were transduced with HaMDR1. HW80 donor bone marrow cells were transduced with HaDHFR. Cells were expanded for 12 days in culture then combined according to hind limb volume. 0.005 vols. expanded cells were competed against 0.25 vols. of fresh competing marrow. MDR1 expanded cells effectively competed against fresh HW80 marrow (Figure 4A, left). DHFR-expanded marrow was completely out competed by fresh C57 marrow (Figure 4A, middle). When MDR1 (C57) was competed against DHFR (HW80) at equal vols. mice reconstituted solely with MDR1 marrow, indicating a much greater stem cell content (Figure 4A, right). The comparison of the bands for the distinctive Hb patterns of C57 and HW80 are shown in Figure 4B. Figure 4C shows the Hb patterns in recipient mice (lanes 1-10) as analyzed by Hb electrophoresis.

Figure 5 shows the secondary CFU-S analysis for HaMDR1 marked primitive hematopoietic cells. At time points from 10 to 24 weeks post-transplant, primary recipients from MDR vs. DHFR competitive repopulation mice (n=6) and MDR 15 were sacrificed and bone marrow cells were injected into secondary recipients. Day 12 CFU-S were harvested and DNA was prepared for Southern blot analysis. DNA was restricted with EcoR1 and probed with an MDR1 specific probe. A band of the correct size was seen in all CFU-S examined (88/88) from 7 individual mice. 56 representative examples are shown. Negative controls included CFU-S from mice transplanted with untransduced bone marrow. All 10 of these (4 shown) did not contain HaMDR1 retroviral DNA.

Figure 6A shows the kinetic analysis of HaMDR1 transduced stem cell expansion. Bone marrow cells were transduced with the HaMDR-1 retrovirus and expanded for the indicated time points. HaMDR cells (0.02 vols/HW80 background) were combined with fresh competed marrow (0.25 vols/C57 background) and injected into lethally irradiated mice. Unexpanded MDR-1 bone marrow (day 0) did not out compete fresh marrow. However, expansion for 3 to 12 days resulted in a progressive increase in engraftment. Figure 6B shows Southern blot analysis of hemoglobin DNA for multilineage engraftment. DNA was prepared from the peripheral blood of mice #20, 21, and 26. DNA was restricted with EcoR1 and probed with a hemoglobin-specific probe. The appropriate bands are indicated by an arrow for both C57 (single) and HW80 (diffuse). The level of lympho-myeloid engraftment determined by DNA analysis correlates very well with the level of engraftment determined in the erythroid lineage by hemoglobin electrophoresis.

Figure 7A shows the kinetics of white blood cell elevation in mice engrafted with expanded bone marrow. The WBC count for all 10 engrafted mice from Figures 6A-6B are shown as they were examined serially. Mice typically had a long latent period of above normal to normal WBC counts followed by a rapid phase observed from 5 to 8 months later. Two mice from this experiment maintain long-term engraftment and continue to have normal WBC counts at time points 8.5 months post-transplant. Figure 7B shows Wright-stained peripheral blood smears from mice displaying an abnormal cell population. A normal mouse smear at the indicated magnifications is

shown (top). The second example demonstrates the most common morphology seen (middle). In a few rare cases, the third blast-like phenotype was seen indicating probable transformation from a myeloproliferative disorder into a leukemia (bottom).

Figure 8 shows the quantitation of Hoechst 33342 SP cells in bone marrow expansion cultures following MDR1 gene transfer. Murine bone marrow cells were prestimulated for 48 hours in the presence of growth factors and then retrovirally transduced with either the HaMDR1 or HaDHFR^{L22Y} vector for another 48 hours. The time point immediately following co-culture was defined as day 0 of expansion. Transduced cells were then grown in suspension cultures for 12 days. Hoechst 33342 staining of BM cells was performed on day 0, 6, and 12 to determine the frequency of SP cells within the expanding cell populations. FACS profiles representative of 3 independent expansion experiments are shown for BM cells transduced with either the HaMDR1 (top panels) or HaDHFR^{L22Y} vectors (bottom panels). On the left, a sample of normal, freshly isolated C57BL/6 BM cells is shown with the SP gate indicated.

Figures 9A-9B show the limiting dilution transplant analysis of MDR1-transduced SP cells isolated from 12 day expansion cultures. HaMDR1-transduced BM cells (C57BL/6 background) were placed into liquid suspension culture and expanded for 12 days. On day 12, the SP cell fraction was isolated by flow cytometry using the sorting gate shown in Fig. 9A. The sorted cells were then injected into lethally irradiated recipient mice at the indicated doses, along with a 2 x 10^5 fresh BM cells (HW80 background). Fig. 9A depicts the results 16 weeks following transplant, peripheral blood leukocyte DNA was prepared and analyzed by PCR for the presence of the HaMDR1 proviral genome. A water-only control and a non-transplanted mouse control (HW80) are shown on the left as negative controls. Transplanted mice received cell doses ranging from 250 to 9400 cells as indicated above the lanes. The numbers 1-18 correspond to individual recipient mice. Fig. 9B depicts the results when the reconstitution was also measured using hemoglobin electrophoresis. Erythroid cells derived from the sorted SP cells are identified by the faster migrating, C57BL/6-derived hemoglobin isoform. The animal numbers are the same as in panel A, and samples from untransplanted C57BL/6 and HW80 mice are shown on the

right. The asterisks indicate samples where there were detectable amounts of C57BL/6-derived hemoglobin.

Figures 10A-10C show the results of the competitive repopulation assay in mice transplanted with equal donor volumes of HaMDR1- and HaDHFR^{L22Y}-transduced BM cells. BM cells from C57BL/6 mice were transduced with the HaMDR1 vector, and HW80 marrow cells with the HaDHFR^{L22Y} vector. Immediately after the transduction, equal donor volumes from each transduced graft were mixed and transplanted into lethally-irradiated recipient mice. Engraftment was monitored by tracking the proportion of donor hemoglobin specific for either the HaMDR1- (lower band) or HaDHFR^{L22Y}- (upper two bands) transduced cells. Hemoglobin electrophoresis gels are shown in Fig. 10A for mice from expt. #2, obtained at 7, 14, and 24 weeks following transplant. These mice were divided into two groups, one receiving two 5-day courses of G-CSF/SCF treatment (left) and one untreated group (right). Each lane represents a sample from a single mouse. The hemoglobin pattern for the C57BL/6 and HW80 controls are shown on the right. Two independent mixing experiments were performed and the data for both of these are graphically shown in Figs.10B-10C. The y-axis represents the percentage of donor engraftment with MDR1-transduced cells as indicated by hemoglobin electrophoresis. Each line represents an individual mouse analyzed serially over time.

Figure 11 depicts the clonality analysis of secondary CFU-S from mice transplanted with HaMDR1-transduced BM cells. Two mice from mixing experiment #1 were killed 24 and 20 weeks after transplant, and bone marrow-derived CFU-S colonies were harvested 12 days after injection into irradiated mice. DNA was prepared from each CFU-S colony and analyzed for vector integration sites by Southern blot analysis. Because the probe is upstream of the 5' EcoRI site in the vector, each band represents a unique integration site within a CFU-S clone. The left panel shows the analysis of 7 clones derived from mouse #12, and the right panel shows 11 clones from mouse #10. DNA from a normal spleen (control) shows a faint endogenous band hybridizing with the MDR1 probe fragment. The DNA ladder is shown on each gel and the ladder marker sizes are indicated on the left. The small arrows in the right panel indicate unique retroviral integration sites in a parent stem cell clone.

Figures 12A-12D depict the P-gp expression and function in producer cells transduced with the HaMDR1Δ34 vector. The monoclonal antibody 4E3 was used to detect cell surface expression of P-gp in GP-E86 cells transduced with the HaMDR1 vector in Fig. 12A or with the HaMDR1Δ34 vector in Fig. 12B. The heavy line represents results from the producer cell lines, while the lighter lines show negative controls using untransduced GP-E86 cells. Molecular pump function was also assayed in these cells using a Rhodamine exclusion assay (HaMDR1 in Fig. 12C, and HaMDR1Δ34 in Fig. 12D). The presence of cells with low fluorescence indicates rhodamine efflux activity due to P-gp expression. Again, the light lines are untransduced negative controls.

Figures 13A-13B show the transduction efficiency of vectors in primary CFU-S. Whole bone marrow cells were transduced either with the HaMDR1Δ34 vector (Fig. 13A, top), or the HaMDR1 vector (Fig. 13B, bottom). The cells were then injected into irradiated recipients, and DNA was later prepared from day 14 CFU-S colonies. Southern blot analysis was performed to detect the proviral genome by digesting with EcoRI and probing with a full-length MDR1 cDNA. Each lane represents a sample from an individual colony. The lane marked by an asterisk is from an untransduced CFU-S colony, which serves as a negative control.

Figures 14A-14D depict the quantitation of SP cell expansion in cultures of cells transduced with the HaMDR1Δ34 vector. BM cells were transduced with either the HaMDR1Δ34 (in Figs. 14A and 14C) or the HaMDR1 vector (14B and 14D), and then expanded in suspension cultures for the indicated time periods. Figs.14A and 14B show SP cell analyses performed after 7 days of expansion. Figs.14C and 14D show a second independent experiment analyzed after 13 days of culture. The percentage of cells in the SP cell gate is indicated in each panel. In experiment 2, propidium iodide was added before flow cytometry analysis to exclude dead cells from the initial gated population.

Figures 15A-15B show the competitive repopulation studies using cells transduced with the HaMDR1Δ34 vector. Fig. 15A shows the hemoglobin electrophoresis of 12-day expanded cells competed against fresh bone marrow cells. C57BL/6 cells were

transduced with either the HaMDR1 vector (left lanes), or with the HaMDR1Δ34 vector (right lanes), and competed against fresh HW80 bone marrow cells at a femur volume ratio of 0.02 transduced to 0.25 fresh. Sixteen weeks after transplant into irradiated recipient mice, hemoglobin electrophoresis was performed to monitor engraftment from the two donor sources. Each lane represents the results from an individual transplanted mouse. Fig. 15B shows the PCR analysis of DNA from peripheral leukocytes of mice transplanted with equal volumes of HaMDR1 and HaMDR1Δ34-transduced marrow. The single primer set was used that flanked the "Δ34 " deletion, and amplified a 692 bp and 590 bp fragment from the HaMDR1 and the HaMDR1Δ34 proviral genomes respectively. From the left, the first lane is from a mock transduced mouse, the second lane from a mouse that received only HaMDR1Δ34-transduced cells, and the third lane from a mouse that received only HaMDR1-transduced cells. All the rest of the lanes are from 2 independent experiments where mice were transplanted using 0.02 femur volumes from each expanded graft. The three mice in the first experiment were analyzed 11 weeks after transplant, and the last 6 lanes are from mice from a second experiment analyzed 18 weeks after transplant.

Figures 16A-16D show the SP cell analyses of BM cells from *mdr1a/mdr1b* knockout mice. Figures 16A-16B show the efflux of Rho 123 in peripheral blood leukocytes from wild type (Fig.16A) and knockout mice (Fig.16B). Figures 16C-16D depict the SP cell analyses of bone marrow cells from wild type (Fig.16C) and knockout mice (Fig.16D). The arrows show the SP cell fraction in each case.

Figures 17A-17C show the results of the treatment of the SP cell phenotype of BM cells from *mdr1a/mdr1b* knockout mice with ABC transport inhibitors. The bone marrow cells were either untreated (Fig.17A), treated with verapamil (Fig.17B) or treated with 2-deoxyglucose (Fig.17C) before and during Hoechst dye staining and efflux. The resulting SP analyses are shown.

Figure 18 shows the RT-PCR analysis of ABC transporter expression in SP Cells from *mdr1a/mdr1b* knockout mice. cDNAs from liver and sorted SP cells were amplified for 35 cycles with the indicated primer sets in the presence of ^{32}P-labeled

dCTP and then resolved by gel electrophoresis. The bands, as indicated, were the size anticipated. No signals were detected in mRNA samples without reverse transcriptase.

Figure19 shows the expression of ABC transporter mRNAs in various sorted hematopoietic populations from normal mice. Expression of *Bcrp1, mrp4, mrp2,* and *mrp1* was assessed by RT-PCR in CD34+ and - subpopulations of c-kit+, Scal+, lin- (KSL) bone marrow cells. Other markers used for sorting are shown above each column, including thymocytes that were double negative (DN) and double positive for CD4 and 8 expression. All RNAs gave equivalent GAPDH mRNA signals.

Figures 20A-20C show the efflux of Hoechst dye in control (Fig. 20A), BCRP (Fig. 20C) and MRP1 (Fig. 20B) retroviral producer cell lines. Cells were incubated with Hoechst dye, and then allowed to efflux in dye-free media for 60 minutes. Cells were then analyzed for Hoechst dye staining by flow cytometry. Parental GPE86 cells and an MRP1 producer line were assayed together with the BCRP line.

Figures 21A-21B show the *ex vivo* expansion of SP cells after transduction with a BCRP retroviral vector. Murine bone marrow cells were transduced with MSCV-BCRP-GFP (Fig. 21B) or a MGMT control vector (Fig. 21A), and then cultured for an additional 7 days in IL3, IL6, and SCF. At the end of the culture period, the expanded population was analyzed for SP cells after Hoechst dye staining. The measured proportion of cells in the SP gate is indicated within each panel.

Figures 22A-22E depict *Brcp1* expression in Rhesus monkey bone marrow SP cells and in muscle-derived SP cells. Figures 22A-22C show an SP analysis f Rhesus bone marrow cells with the sorting gates used for the SP (R2) and non-SP cells (R3). RNA was amplified by RT-PCR from 2000 sorted SP cells and 10,000 non-SP cells at the indicated number of cycles using β-actin or BCRP primers, as indicated. Roughly equivalent points on the β-actin amplification curve are shown for both samples. Figures 22D-22E show murine skeletal muscle cells which were stained with Hoechst dye and SP cells that were isolated by sorting. RNA was prepared from the sorted SP cells, and analyzed for expression of *Brcp1* and β2 microglobulin by RT-PCR.

Figures 23A-23B show *Bcrp1* expression in pediatric AML blasts. RNA obtained from bone marrow blast cells from four individuals with AML was amplified by RT-PCR using primers for BCRP (Figure 23A) or β-actin (Figure 23B). Cycle numbers are indicated above each lane, and the AML phenotype and blast percentage are also shown.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods of performing *ex vivo* expansion of gene-modified hematopoietic stem cells which are useful for many applications including bone marrow transplantation, and *ex vivo* gene therapy. In addition, the present invention provides methods of engrafting the gene-modified hematopoietic stem cells of the present invention into animals, including for bone marrow transplantation and *ex vivo* gene therapy. Therefore the present invention provides methods of treating an animal in need of treatment for a hematopoietic stem cell deficiency using a method of engrafting the expanded gene modified hematopoietic stem cells of the present invention. In one such embodiment the hematopoietic stem cell is transduced *ex vivo* with a nucleic acid encoding a transmembrane efflux pump, *e.g.* MDR1. The transduced hematopoietic stem cell (*i.e.*, a gene-modified hematopoietic stem cell) is expanded and then engrafted into the animal. Preferably the hematopoietic stem cell used is matched with the recipient animal to minimize and/or prevent host rejection. Thus, the hematopoietic stem cell is preferably obtained from the animal in need of treatment, and then after being transduced with a nucleic acid encoding MDR1 and expanded, the resulting gene-modified hematopoietic stem cell is placed back into the animal. The treatments as described herein may be used for any hematopoietic stem cell deficiency including that due to radiation therapy and/or chemotherapy, *e.g.*, as used in cancer treatments. One particular advantage for treating a hematopoietic stem cell deficiency due to chemotherapy with a method of the present invention is that a gene-modified hematopoietic stem cell transduced with a nucleic acid encoding MDR1 will also be protected from the chemotherapeutic and its adverse effects. Therefore, the engrafting of the gene-modified hematopoietic stem cell into the recipient animal can be performed concomitantly with the chemotherapy.

The *ex vivo* gene therapy methodology of the present invention can be used for treating any disorder (particularly a genetic disorder) that involves a defect in a cell derived from a hematopoietic stem cell including but not limited to the treatment of thalassemia (*e.g.*, with an expanded modified hematopoietic stem cell encoding human β-globin), Gaucher's disease (*e.g.*, with an expanded modified hematopoietic stem cell encoding glucocerebrosidase), sickle cell anemia, and leukemia.

The present invention further provides the expanded gene-modified hematopoietic stem cells used and/or produced by such methods. Such expanded gene-modified hematopoietic stem cells can also contain a second heterologous gene.

In addition, the ability of expanding the otherwise rare hematopoietic stem cells provided by the present invention provides a source of hematopoietic stem cells which is large enough in quantity to allow standard biochemical analysis to be performed on this relatively unstudied cell type. Indeed, the present invention results in the capability of the expanding of human hematopoietic stem cells that is greatly increased from the maximum of about four-fold expansion taught in the prior art. Thus, the present invention provides a means for performing facile assays for identifying factors involved in the regulation of the proliferation *versus* differentiation of hematopoietic stem cells, particularly human hematopoietic stem cells. Such assays, for example, can be based on the experimental conditions taught herein and the administration of fractionated cellular extracts. Naturally occurring factors can be identified by such assays and then isolated by convention biochemical procedures. Alternatively, chemical libraries and/or phage libraries can be used in an analogous drug screening assays. These naturally occurring factors and drugs can then be used to manipulate the fate of hematopoietic stem cells initially *in vitro* and eventually *in vivo*. Indeed, currently there are no known factors which specifically lead to the proliferation of hematopoietic stem cells in the absence of differentiation.

In Example 1, below, bone marrow cells were transduced with a Harvey (Ha)/MDR1 retrovirus and expanded for 12 days in the presence of interleukin (IL)-3, IL-6, and stem cell factor (SCF). Long-term engraftment in non-irradiated mice was observed after transplantation of the cells which were transduced with Ha/MDR1. To compare

relative repopulating activities of expanded vs. unexpanded cells, competitive repopulation experiments in irradiated recipients were performed. These results showed at least a 10-fold increase in the absolute number of repopulating cells relative to fresh untransduced marrow. The results demonstrate that MDR1 overexpression allows dramatic cytokine-driven expansion of hematopoietic stem cells *in vitro*.

In Example 2 it is shown that MDR1-mediated stem cell expansion is associated with an increase in "side population" (SP) stem cells, defined by Hoechst dye staining. Transduction of murine bone marrow cells with an MDR1 retroviral vector resulted in an almost 2 log increase in SP cell numbers over 12 days in culture, while there was a rapid loss of SP cells from control cultures. Stem cell amplification was not limited to *ex vivo* expansion cultures, but was also evident when MDR1-transduced cells were directly transplanted into irradiated mice. In these cases, stem cell expansion was associated with relatively high vector copy numbers in stem cell clones. As previously indicated herein, some cases were associated with a characteristic myeloproliferative syndrome. A functionally inactive MDR1 mutant cDNA was used to show that P-gp pump function was required both for amplification of phenotypically-defined SP cells, and functionally-defined repopulating cells. These results further support the concept that ABC transporter function can have important effects on hematopoietic stem cell development.

The universal presence of transporter activity and expression in hematopoietic stem cell, and the generally observed down regulation of transporter expression that occurs with differentiation, can be explained by a functional role for ABC transporters in promoting self-renewal outcomes. Based on Example 1 below, showing that enforced MDR1 expression causes stem cell expansion in a variety of settings, and recently published studies showing that MDR1 expression can inhibit apoptosis in hematopoietic cell lines [Johnstone *et al.*, *Blood* **93**:1075-1085 (1999)], MDR1 expression appears to promote stem cell self-renewal by causing a relative block to apoptosis during stem cell division. This function could reflect the normal role for endogenous transporter expression in stem cells, as well as indicate a role for dysregulated transporter expression in leukemogenesis. Interestingly, the present

invention discloses that the MDR1 gene is not necessary for dye efflux in HSCs, or for the SP phenotype.

As shown herein, the *Bcrp* gene is expressed at relatively high levels both in primitive CD34- murine HSCs and in SP cells from the bone marrow. In contrast, the expression of other known ABC transporters in the highly enriched CD34- stem cell population is low to absent. A retroviral vector expressing the human BCRP cDNA has been constructed as described below to study the functional properties of BCRP. Fibroblasts expressing this vector gain the capacity to efflux Hoechst dye, a prerequisite property for establishment of the SP phenotype. Furthermore, when primary bone marrow cells are transduced with the BCRP vector, there is a large expansion of SP cells over time in culture. SP cells from the muscle also express *Bcrp* at high levels. Indeed, BCRP mRNA expression is highly restricted in normal tissues, and cannot be detected in adult tissues at the Northern blot level. Furthermore, as disclosed herein, *Bcrp* expression is relatively restricted to the hematopoietic stem cell compartment in mice. These data indicate that *Bcrp* expression may be a universal marker for stem cells from various organs, and may be the critical molecule for conferring the dye efflux phenotype to stem cells. Therefore, the present invention provides a method of identifying stem cells by their expression of BCRP. Such identification can then be used to isolate the stem cells.

In addition, both MDR1 and BCRP appear to be able to promote stem cell amplification and self-renewal by decreasing the probability of apoptosis as an outcome of stem cell division. HSCs therefore, appear to require expression of at least one of these transporters to sustain normal hematopoiesis over time.

Since tightly regulated expression of ABC transporters appears to be required for normal hematopoiesis, it follows that dysregulated expression could lead to hematopoietic abnormalities, or even contribute to leukemogenesis. Such effects in mice transplanted with MDR1 vectors are demonstrated below, in an experimental setting where expression of P-gp is abnormally increased in primitive cells and proper developmental regulation of P-gp is over-ridden. Indeed, when the expression of P-gp was abnormally increased, a significant proportion of transplanted mice developed a

myeloproliferative syndrome characterized by peripheral blood leukocytosis, an increase in immature myeloid forms in the circulation, and significant degrees of splenomegaly (*see* Example 1 below). This syndrome developed slowly, with the first cases being noted two to three months after transplant, and with the incidence increasing to about 50% by six to nine months. The delayed pattern observed is consistent with the acquisition of a second genetic lesion that is required for development of the syndrome. This second lesion seems to be related to the prior degree of stem cell expansion conferred by the MDR1 vector because mice transplanted with *ex vivo* expanded cells showed a higher incidence of myeloproliferation than mice transplanted with freshly transduced, unexpanded cells (Example 2 below).

Furthermore, early myeloproliferation is associated with a relatively high vector copy number in transduced stem cell clones (*see* Example 2 below), indicating that development of the syndrome may be dependent on a high threshold level of expression of the transferred MDR1 gene. Altogether, these data show that dysregulated MDR1 expression can contribute to leukemogenesis, and they also indicate that dysregulated expression of other ABC transporters may also lead to disordered hematopoiesis.

In addition, expression of other uncharacterized ABC transporters occurs in acute myelogenous leukemia (AML) with significant frequency. For example, dysregulated BCRP expression is likely to be involved in at least some of these cases, based on its known capacity for dye efflux, and its tightly regulated expression pattern during normal myeloid development (*see* Example 3). Furthermore, given the capacity of BCRP to confer resistance to anthracyline drugs [Miyake *et al.*, *Cancer Res.* **59**:8-13 (1999)], BCRP expression may directly confer resistance to AML induction chemotherapy. Indeed, BCRP expression in AML blasts appears to be associated with a drug resistant phenotype and thereby predict a poor prognosis. Therefore, the present invention also provides a method of diagnosing/prognostigating pediatric patients with AML, *e.g.*, by examining/monitoring blast cells from such pediatric patients.

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "hematopoietic stem cell" is a pluripotent cell that is able to either replicate itself with self-renewal divisions or to differentiate along a number of pathways and thereby generate erythrocytes, granulocytes, monocytes, mast cells, lymphocytes, and megakaryocytes. These stem cells occur with a frequency of about 1 stem cell per 10^4 bone marrow cells.

A "heterologous gene" as used herein is a gene that is introduced into a stem cell (e.g., a hematopoietic stem cell) through a molecular biological manipulation. As defined herein, this molecular biological manipulation is made such that the heterologous gene is inserted into the stem cell. The heterologous gene need not be expressed in the stem cell as long as it is expressed in the progeny of the stem cell. The coding sequence of the heterologous gene is operatively linked to an expression control sequence. Generally a heterologous gene is first placed into a vector. The heterologous gene is not necessarily naturally contained by the vector, though a heterologous gene can encode a protein that is native to the stem cell. For example, the heterologous gene can encode a functional protein and be used in *ex vivo* gene therapy to replace the corresponding defective gene in a stem cell, *e.g.*, an hematopoietic stem cell. The heterologous gene will usually be flanked by DNA that does not flank the genomic DNA in the genome of the source organism. Alternatively, the heterologous gene may not be naturally found in the stem cell, such as the gene for human MDR1 introduced into a murine hematopoietic stem cell.

A cell has been "transduced" by a heterologous gene such as the MDR1 gene (*i.e.*, a nucleic acid encoding MDR1), when the gene has been introduced inside the cell and the coding sequence of the gene is operatively linked to an expression control sequence. The transducing gene is carried by a vector and the gene may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. A stably transduced cell is one in which the transducing gene has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the cell to

establish cell lines or clones comprised of a population of daughter cells containing the transducing gene. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

As used herein a "gene-modified hematopoietic stem cell" is a hematopoietic stem cell that has been transduced by a heterologous gene. A gene-modified hematopoietic stem cell transduced with a nucleic acid encoding MDR1 (the *mdr1* gene) is exemplified below.

As used herein the "expansion" of an hematopoietic stem cell indicates that there is an increase in the absolute number of hematopoietic stem cells, *i.e.*, during the culturing of the cells. Analogously, an hematopoietic cell that has undergone such expansion has been "expanded".

As used herein "engrafting" a stem cell, preferably an expanded hematopoietic stem cell, means placing the stem cell into an animal, *e.g.*, by injection, wherein the stem cell persists *in vivo*. This can be readily measured by the ability of the hematopoietic stem cell, for example, to contribute to the ongoing blood formation.

As used herein an "ABC transporter" is used in the conventional sense and is used to describe a protein that is a transport ATPase. ABC transporters are members of a large family of transport proteins that are ATP-dependent. The name is derived from a highly conserved ATP-binding cassette contained by all of the members. [*See*, Alberts *et al., Molecular Biology of the Cell*, 3rd edition, Garland Publishing Inc. (New York) Pages 519-522 (1994)]. MDR1 and BCRP are two transmembrane efflux pumps that are part of the family of ABC transporters.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; *i.e.*, capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. The term "vector" can also refer to a recombinant virus or defective virus containing a replicon to which another DNA segment may be attached.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA).

A "coding sequence" is a nucleic acid sequence which can be reverse transcribed (*i.e.*, when part of a retroviral vector) and/or transcribed and then translated into a polypeptide *in vitro* and/or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

A nucleic acid sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and/or translation of that nucleic acid sequence. The term "operatively linked" includes having an appropriate start signal (*e.g.*, ATG) in front of the nucleic acid sequence to be expressed and maintaining the correct reading frame to permit expression of the nucleic acid sequence under the control of the expression control sequence and

production of the desired product encoded by the nucleic acid sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal for example, such a start signal can be inserted in front of the gene.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma, adenovirus, herpes virus and other sequences known to control the expression of genes of mammalian cells, and various combinations thereof.

In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its

controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence combinations that will express the heterologous genes used in the present invention.

A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes. For instance, alpha-factor, a native yeast protein, is secreted from yeast, and its signal sequence can be attached to heterologous proteins to be secreted into the media (See U.S. Patent 4,546,082, EPO 0 116 201, publication date 12 January 1983; U.S. Patent Application Serial No. 522,909, filed 12 August 1983). Further, the alpha-factor leader and its analogs have been found to secrete heterologous proteins from a variety of yeast, such as Saccharomyces and Kluyveromyces, (EPO 88312306.9 filed 23 December 1988; U.S. Patent Application Serial No. 139,682, filed 30 December 1987, and EPO Publication No. 0 301 669, publication date 1 February 1989).

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous

solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, and most preferably prevent and/or treat, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

Vectors

According to the present invention, the vector for *ex vivo* administration of the gene encoding an ABC transporter such as MDR1 or BCRP (*i.e.*, a nucleic acid encoding MDR1 or BCRP respectively) and/or an alternative heterologous gene can be introduced *via* any strategy. Vectors can be introduced to transduce the desired host cells *ex vivo* by methods known in the art, *e.g.*, transfection, electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, using a viral vector, with a DNA vector transporter, and the like. Alternatively, the vector can be introduced by lipofection.

Viral vectors are commonly used for *ex vivo* targeting and therapy procedures; these include DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art [*see, e.g.*, Miller and Rosman, *BioTechniques* **7**:980-990 (1992)]. DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. In addition, different viral vectors may exhibit specificity for one or another cell type. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt *et al.*, *Molec. Cell. Neurosci.* **2**:320-330 (1991)], defective herpes virus vector lacking a glyco-protein L gene [Patent Publication RD 371005 a], or other defective herpes

virus vectors [International Patent Publication No. WO 94/21807, published September 29, 1994; International Patent Publication No. WO 92/05263, published April 2, 1994]; an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al. [*J. Clin. Invest.* **90**:626-630 (1992); *see also* La Salle *et al.*, *Science* **259**:988-990 (1993)]; and a defective adeno-associated virus vector [Samulski et al., *J. Virol.* **61**:3096-3101 (1987); Samulski *et al.*, *J. Virol.* **63**:3822-3828 (1989); Lebkowski *et al.*, *Mol. Cell. Biol.* **8**:3988-3996 (1988)]. Herpes virus vectors are preferred for dendritic cells.

In another embodiment, the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson et al., U.S. Patent No. 5,399,346; Mann *et al.*, 1983, Cell 33:153; Temin *et al.*, U.S. Patent No. 4,650,764; Temin *et al.*, U.S. Patent No. 4,980,289; Markowitz et al., 1988, J. Virol. 62:1120; Temin *et al.*, U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty *et al.*; and Kuo *et al.*, 1993, Blood 82:845. International Patent Publication No. WO 95/07358 describes high efficiency transduction of primary B lymphocytes. In a specific embodiment, exemplified below, a Harvey murine retroviral vector is used to transduce hematopoietic stem cells. Retroviral vectors can be constructed to function as infectious particles or to undergo a single round of transfection. In the former case, the virus is modified to retain only those genes responsible for packaging and replication and to express the heterologous gene. Non-infectious viral vectors are prepared to destroy the viral packaging signal, but retain the structural genes required to package the co-introduced virus engineered to contain the heterologous gene and the packaging signals. Thus, the viral particles that are produced are not capable of producing additional virus.

For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* or *ex vivo* transfection of a gene encoding a marker [Felgner *et. al.*, *Proc.Natl.Acad.Sci.USA* **84**:7413-7417 (1987); *see* Mackey *et al.*, *Proc.Natl.Acad.Sci.USA* **85**:8027-8031 (1988); Ulmer *et al.*, *Science* **259**:1745-1748 (1993)]. The use of cationic lipids may promote encapsulation of negatively charged

nucleic acids, and also promote fusion with negatively charged cell membranes [Felgner and Ringold, *Science* **337**:387-388 (1989)].

It is also possible to introduce the vector as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter [*see, e.g.*, Wu *et al.*, *J. Biol. Chem.* **267**:963-967 (1992); Wu and Wu, *J. Biol. Chem.* **263**:14621-14624 (1988); Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990; Williams et al., *Proc.Natl.Acad.Sci. USA* **88**:2726-2730 (1991)].

The present invention includes vectors containing a gene (*i.e.*, a nucleic acid) encoding a transmembrane efflux pump, *e.g.*, MDR1 or BCRP. Also included are truncated forms, analogs and derivatives of the transmembrane efflux pump, *e.g.*, MDR1 that have essentially the same or improved functional activity as MDR1, for example. Therefore, the production and use of derivatives and analogs related to MDR1 or BCRP, for example, are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type MDR1 protein.

In particular, MDR1 or BCRP derivatives, for example, can be made by altering encoding nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity as it relates to the present invention, relative to the native MDR1 or BCRP, for example.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as an ABC transporter gene, may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the ABC transporter genes which are altered by the substitution of different codons that encode the same amino acid

residue within the sequence, thus producing a silent change. Likewise, the ABC transporter derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of the ABC transporter protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Additionally, the nucleic acid sequence encoding the transmembrane efflux pump, such as BCRP, can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity of the mutated BCRP gene product, for example. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis [Hutchinson, *et al.*, *J. Biol. Chem.* **253**:6551(1978.); Zoller and Smith, *DNA* **3**:479-488 (1984); Oliphant *et al.*, *Gene* **44**:177 (1986); Hutchinson *et al.*, *Proc.Natl.Acad.Sci.USA* **83**:710 (1986), use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis [*see* Higuchi, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70 (1989)].

Expression vectors containing a transmembrane efflux pump gene, such as the MDR1 gene, inserts can be identified by many ways including : (a) PCR amplification of the desired plasmid DNA or specific mRNA, (b) nucleic acid hybridization, (c) presence

or absence of "marker" gene functions, and (d) expression of inserted sequences. In the first approach, the nucleic acids can be amplified by PCR with incorporation of radionucleotides or stained with ethidium bromide to provide for detection of the amplified product. In the second approach, the presence of a MDR1 gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted MDR1 gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, β-galactosidase activity, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. In the fourth approach, recombinant expression vectors can be identified by assaying for the activity and/or presence of the MDR1 gene product expressed by the recombinant.

Promoters

According to the present invention, the gene encoding an ABC transporter such as MDR1 or BCRP, and/or a second heterologous gene can be under the control of any promoter. In a specific embodiment, the human cytomegalovirus (CMV) immediate early promoter is used to effect transient expression of the ABC transporter. Alternatively, an inducible promoter can be used. However, the present invention contemplates use of any promoter to control expression of the ABC transporter. Selection of the promoter depends on the desired use. For example, expression of the ABC transporter may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host or host cell selected for expression. Promoters which may be used to control the ABC transporter gene expression include, but are not limited to, the SV40 early promoter region [Benoist and Chambon, *Nature* **290**:304-310 (1981)], the promoter contained in the 3' long terminal repeat of Rous sarcoma virus [Yamamoto *et al.*, *Cell* **22**:787-797 (1980)], the herpes thymidine kinase promoter [Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* **78**:1441-1445 (1981)], the regulatory sequences of the metallothionein gene [Brinster *et al.*, *Nature* **296**:39-42 (1982)]; and using a transcriptional control region such as the beta-globin gene control region which is active in myeloid cells [Mogram *et al.*, *Nature* **315**:338-340 (1985); Kollias *et al.*, *Cell* **46**:89-94 (1986)]. Alternatively,

expression of the the ABC transporter gene can be under control of an inducible promoter, such as metallothionein promoter, which is induced by exposure to heavy metals.

Bone Marrow Transduction

Bone marrow cells can be obtained from any number of sources from an animal, including a human subject. For example, the cells can be harvested from iliac bone marrow. Alternatively, hematopoietic stem cells can be obtained from umbilical chord cells. Another source for hematopoietic stem cells is from circulating fetal blood cells. In addition, a human subject, for example, can be treated with a cytotoxic drug and/or a hematopoietic stem cell stimulating cytokine (*e.g.*, G-CSF). Mononuclear cells can then be collected by leukophoresis and the hematopoietic stem cells can be isolated from the peripheral blood cells by their selective binding to an antibody raised against CD34.

In Example 1 below, bone marrow cells were flushed from the hind limbs of a laboratory animal and prestimulated for 48 hours in an appropriate medium. Dulbecco's modified essential medium supplemented with 15% fetal bovine serum, 100 units/ml penicillin, and 100 ng/ml streptomycin (P/S; Gibco-BRL) was also used in Example 1 below. Growth factors can also be included in the suspension culture at the appropriate concentration. Following prestimulation cells can be co-cultured on irradiated retroviral producer cell lines (e.g., ecotropic producer cell lines for mice and amphotropic producer cell lines for humans) for 48 hours in the presence of the same growth factor combination but also with added 6 mg/ml polybrene (Sigma) to enhance transduction.

Ex Vivo Culture and Expansion of Myeloid Progenitors

After transduction, cells can be cultured. In Example 1 below, culturing was performed in the presence of interleukin-3, interleukin-6, and stem cell factor. Any other cytokine which supports the proliferation of hematopoietic stem cells could be used, including but not limited to GM-CSF, G-CSF and FLT-3 ligand. It is preferred that when the hematopoietic stem cell is a human cell and/or the recipient is a human subject that the cytokines used also be the human homolog.

Cells are typically resuspended at 1 x 10^6 cells/ml every 3-6 days for at least 12 days of expansion. Aliquots of cells can be removed for CFU-C analysis at various time points. The percentage of drug-resistant progenitors can calculated by plating cells in methylcellulose (Stem Cell Technologies) for example, in the presence of selective concentrations of drugs. The percentage of transduced cells are generally found to remain constant throughout expansion.

Non-irradiated Recipient Bone Marrow Transplants

For bone marrow transplants into non-irradiated recipients, mice can receive from 1 to 10 daily intravenous injections with a vector containing a nucleic acid encoding MDR1, *e.g.*, a total of 20 - 40 x 10^6 cells for the 5 day period exemplified below. (Humans can receive from 1 to 20 such daily intravenous injections, preferably 5 to 10 daily intravenous injections). Following a five day treatment course in Example 1 below, the presence of a donor marker protein, Hb as exemplified below, can be monitored in recipient animal (as exemplified below the monitoring began at one week after the last injection), and then followed for as long as appropriate, (8 to 14 months in Example 1 below).

Transduction of Murine Bone Marrow Cells with ABC Transporter Vectors

HSC expansion, the development of abnormal myeloproliferation and overexpression, of ABC transporters: Retroviral vectors that express ABC transporters such as those constructed to encode BCRP and MRP4 can be used to transduce murine bone marrow cells. These transduced grafts can then be evaluated for stem cell expansion in competitive repopulation studies to determine if HSCs are amplified *in vitro* or *in vivo*. Transplanted mice can be followed over time for evidence of abnormal peripheral blood counts and myeloproliferation. ABC transporters are identified that can efflux Hoechst dye and mediate the SP cell phenotype. The cDNAs that encode the ABC transporters can then be tested in the retroviral vector system as described herein.

Resistance to apoptosis and/or differentiation: Murine bone marrow cells can be transduced with the HaMDR1sc vector, and grown for 6 days in culture in the presence of cytokines and serum. Then, the cells can be replated in media that

contains no serum or cytokines to induce apoptosis. Annexin and tunnel staining can be performed to determine the number of cells undergoing apoptosis, and the survival of clonogenic cells under apoptosis-inducing conditions can be determined by colony growth in methycellulose-containing media. When an anti-apoptotic effect is demonstrated, the mechanism by which apoptosis is inhibited can be determined. One such method includes looking for the activation of members of the caspase pathway, and determining the distribution of phosphotidylserine in the cell membrane of transduced, sorted cells. When no effects on apoptosis are observed, it can be determined whether ABC transporter expression is leading to an accumulation of HSCs by inducing a differentiation block. FDCP-Mix cells can be transduced with HaMDR1sc and evaluated for changes in cytokine-induced differentiation. Primary murine HSCs can also be studied by evaluating transduced and control cultures for the number of lineage -, CD34-, kit +, sca1+ cells in cultures at various time points in culture. Loss of cells bearing this primitive stem cell phenotype has been reported using normal cells, and is thought to be due to differentiation of HSCs in culture. Any vector comprising an ABC transporter can be evaluated in this way including the HaMDR1 vector and vectors comprising BCRP or MRP4. The results can also be re-evaluated in human bone marrow CD34+ cells using an RD18-derived HaMDR1sc vector, for example.

Defining the downstream changes in gene regulation associated with MDR1-mediated HSC expansion: The ability to isolate expanding stem cells in MDR1-transduced cultures allows the evaluation of changes in gene expression that are associated with stem cell expansion. Thus, murine bone marrow cells can be transduced, and expanded in culture in parallel with mock-transduced cells. After 6 days of expansion, SP cells can be isolated by cell sorting from the transduced population. SP cells generally are not expected in the mock-transduced population at after 6 days, so as control population for subtractive analysis, Sca1-, lineage negative cells, for example, can be isolated from the cultured mock population. RNA can be isolated from both populations, and analyzed by hybridization with matrix-based gene arrays. RNA can be isolated to make suitable fluorescent probes. Alternatively, a sensitive PCR-based subtraction method can be used to identify genes that are upregulated in the transduced, expanding stem cells. Differentially expressed products can be

sequenced, and used to generate probes for further expression in normal stem cell populations. Genes encoding upregulated and downregulated mRNAs can be selected and cloned, and functionally analyzed in overexpression experiments using retroviral vectors similar to those described herein.

Antibodies to the ABC Transporters of the Present Invention

According to the present invention, ABC transporters as produced by a recombinant source, or through chemical synthesis, or an ABC transporter isolated from a natural source; and derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize an ABC transporter such as BCRP. Although exemplified for BCRP, the methodologies regarding making antibodies, as discussed below, are applicable for all of the ABC transporters of the present invention. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric including humanized chimeric, single chain, Fab fragments, and a Fab expression library.

In a particular embodiment an antibody is raised to an external epitope of BCRP. In a particular embodiment the epitope is derived from the extracellular portion of BCRP. Such an antibody can be used to sort living cells on a flow cytometer. These antibodies can be used, for example, to sort hematopoietic cells based on BCRP (*bcrp)* expression. Such antibodies also may be used to detect BCRP as a marker for repopulating activity.

The anti-BCRP antibodies of the invention may be cross reactive, that is, they may recognize a BCRP derived from a different source, *e.g.*, an anti-human BCRP antibody may recognize both human and mouse BCRP. Polyclonal antibodies have greater likelihood of cross reactivity. Alternatively, an antibody of the invention may be specific for a single form of a BCRP, such as the hBCRP having the amino acid sequence of SEQ ID NO:10.

Various procedures known in the art may be used for the production of polyclonal antibodies to BCRP, for example, or derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with the BCRP, or a

derivative (*e.g.*, or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, the BCRP can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward the BCRP, or analog, or derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein [*Nature,* **256**:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor *et al., Immunology Today,* **4**:72 (1983); Cote *et al., Proc. Natl. Acad. Sci. USA,* **80**:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)]. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology [PCT/US90/02545]. In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison *et al., J. Bacteriol.*, **159**:870 (1984); Neuberger *et al., Nature,* **312**:604-608 (1984); Takeda *et al., Nature,* **314**:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific for a BCRP together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. Such human or humanized chimeric antibodies are preferred for use in therapy of human diseases or disorders (described *infra*), since the human or humanized antibodies are much less likely than xenogenic antibodies to induce an immune response, in particular an allergic response, themselves. In a particular embodiment, the BCRP-expressing producer cells of the present invention are used to raise monoclonal antibodies to external cell surface epitopes. Antibody producer

clones can be screened for differential staining of producer cells versus their parental packaging cells.

According to the invention, techniques described for the production of single chain antibodies [U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted to produce for example, BCRP-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse *et al., Science,* **246**:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a BCRP, or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab′ fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of BCRP, one may assay generated hybridomas for a product which binds to the BCRP

fragment containing such epitope and choose those which do not cross-react with BCRP. For selection of an antibody specific to a BCRP from a particular source, one can select on the basis of positive binding with BCRP expressed by or isolated from that specific source.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the BCRP, *e.g.*, for Western blotting, imaging BCRP *in situ*, measuring levels thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned herein or known in the art. In a specific embodiment, antibodies that agonize or antagonize the activity of BCRP can be generated. Such antibodies can be tested using the assays that measure the drug pumping ability of BCRP, for example.

The antibodies to the ABC transporters can be labeled. Suitable labels include enzymes, fluorophores (*e.g.*, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu^{3+}, to name a few fluorophores), chromophores, radioisotopes, chelating agents, dyes, colloidal gold, latex particles, ligands (*e.g.*, biotin), and chemiluminescent agents. In the instance where a radioactive label, such as the isotopes ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{125}I, and ^{131}I, are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or applied stimulation, *e.g.* ultraviolet light to promote fluorescence. Among examples of colored labels, which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734); dye sole particles such as described by Gribnau *et al.* (U.S. Patent 4,373,932) and May *et al.* (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559 and 0 281 327); or dyes

encapsulated in liposomes as described by Campbell *et al.* (U.S. Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a luminescent moiety. In addition to these direct labeling devices, indirect labels comprising enzymes can also be used according to the present invention. Various types of enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, **70:**419-439 (1980) and in U.S. Patent 4,857,453.

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase. In addition, an antibody can be modified to contain a marker protein such as green fluorescent protein as described in U.S. Patent No. 5,625,048 filed April 29, 1997, WO 97/26333, published July 24, 1997 and WO 99/64592 all of which are hereby incorporated by reference in their entireties. Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

In another embodiment, a phosphorylation site can be created on an antibody of the invention for labeling with ^{32}P, *e.g.*, as described in European Patent No. 0372707 (application No. 89311108.8) by Sidney Pestka, or U.S. Patent No. 5,459,240, issued October 17, 1995 to Foxwell *et al.*

Antibodies also can be labeled by metabolic labeling. Metabolic labeling occurs during *in vitro* incubation of the cells that express the protein in the presence of culture medium supplemented with a metabolic label, such as [^{35}S]-methionine or [^{32}P]-orthophosphate. In addition to metabolic (or biosynthetic) labeling with [^{35}S]-methionine, the invention further contemplates labeling with [^{14}C]-amino acids and [^{3}H]-amino acids (with the tritium substituted at non-labile positions).

Stem Cell Purification

After an appropriate antibody is identified, mouse bone marrow cells, for example, can be depleted of lineage positive cells, and the lin – cells can be sorted for BCRP expression. Competitive repopulation assays can then be used to demonstrate the

enrichment of stem cell activity in the BCRP-expressing fraction. BCRP sorting experiments can also be performed in lin-, ckit+, sca1+ cells, and in CD34- cells to determine if these populations can be further enriched for stem cell activity. An analogous procedure can be performed using an alternative cell source, *e.g.*, human cord blood cells.

Thus, BCRP sorting experiments can be performed using Lin-, CD34- cells, as well as CD34+, 38- cells to determine the amount that BCRP expression enriches for repopulating activity in these stem cell populations. Sorted cells then can be injected into NOD/SCID mice in limiting dilution analyses to quantify the stem cell frequencies in these populations. This procedure can be repeated using bone marrow cells and cytokine-mobilized peripheral blood stem cells to demonstrate the utility of the procedure in various clinical stem cell sources. For example, SP cells were isolated from mouse muscle satellite cells and RT-PCR and FACS analysis was used to demonstrate that *Bcrp* is also expressed in these cells (*see* Example 3 below). Reconstitution studies can also be performed using sorted *bcrp*-expressing muscle cells. Sorted donor cells can be identified after transplant using a GFP-transgenic mouse line for donor cells, for example, and analyzing recipients for GFP+ SP cells in the muscle.

Therefore, the present invention provides a functional basis for identifying SP stem cells, and furthermore, provides a new way to isolate stem cells both for research and clinical applications. For example, the present invention provides a method of isolating stem cells using an anti-BCRP antibody. These stem cells can originate from any tissue that contains stem cells including from bone marrow cells, muscle cells and even brain cells. Any method that allows the separation of cells that can be distinguished by their ability to bind a particular antibody can be employed. For example, to isolate hematopoietic stem cells, bone marrow cells can be obtained from an animal subject, (preferably a human). Single cell suspensions can then be prepared. An anti-BCRP antibody can be incubated with the cells and the cells can be isolated using standard cell sorting methodology *e.g.*, by fluorescent cell sorting [Bhatia *et al.*, *Nat. Med.* **4**:1038-1045 (1998)]. In a related embodiment, muscle stem cells can be isolated from a muscle cell sample [Gussoni *et al.*, Nature 401:390

(1999)]. Alternatively, stem cells can be distinguished from non-stem cells by the specificity of the drug-pumping activity of BCRP.

Antisense and Gene Targeting

Given the highly specific expression of *Bcrp1* in hematopoietic stem cells, and the association of enforced ABC transporter expression with stem cell expansion, either the *Bcrp1* and/or *mdr1a/b* genes appear to be required for normal stem cell function. Furthermore, the functional activity of an ABC transporter, and in particular BCRP or MRR1 can be evaluated with transgenic, knockout, or knockin animals. Therefore, the present invention provides transgenic, knockin, and knockout animals. In one embodiment of this type the knockin animal is a mouse. In another embodiment the animal is a knockout mouse. One embodiment comprises a disruption in an endogenous allele encoding BCRP, which prevents the expression of functional BCRP from that individual allele. In another embodiment, the disruption is in both endogenous alleles that encode BCRP, preventing the knockout animal from expressing functional endogenous BCRP. Although a transgenic/knockin /knockout mouse is preferred other rodents such as rats and rabbits, or mammals such as pigs, goats, sheep, and monkeys can also be used.

The present invention also includes non-human transgenic or knockin animals that comprise cells that express an ABC transporter variant of the present invention. For example, a mouse comprising the HaMDR1Δ34 described in the Examples below. Such a transgenic or knockin animal can be used as a control, for example when identifying and testing drugs that can be useful treating leukemia for example. Thus the transgenic, knockin, and knockout animals of the present invention can be used in drug screens and the like. Cells from the transgenic, knockin and knockout mice are also part of the present invention, as are cells that are made *in situ* to overexpress or alternatively, to not express the ABC transporters of the present invention.

The *ABC Transporter* genes such as the *Bcrp* gene also can be used in complementation studies employing transgenic mice. Transgenic vectors, including viral vectors, or cosmid clones (or phage clones) corresponding to the wild type locus of candidate gene, can be constructed using the isolated *Bcrp* gene. Cosmids may be

introduced into transgenic mice using published procedures [Jaenisch, *Science*, **240**:1468-1474 (1988)]. In a particular embodiment, a mouse *Bcrp* gene can be placed into a transgenic or knockin mouse and compared with a wild-type mouse. Similarly, transgenic or knockin animals other than mice may also be generated and used.

A transgenic or knockin animal can thus be prepared that expresses a recombinant BCRP or a fragment thereof. Such transgenic animals can be obtained through gene therapy techniques described above or by microinjection of a nucleic acid [such as a bacterial artificial chromosome (BAC) that encodes a BCRP variant] for example, into an embryonic stem cell or an animal zygote. Microinjection of BACs has been shown to be successful in a number of animals including rats, rabbits, pigs, goats, sheep, and cows [in *Transgenic Animals Generation and Use ed.*,L.M. Houdebine, Harwood Academic Publishers, The Netherlands (1997)]. Methods of constructing BACs [or other DNAs such as bacteriophage P1 derived artificial chromosomes (PACs)] that encode specific nucleic acids through homologous recombination have recently been described in great detail [Heintz *et al.*, PCT/US98/12966, (1998) the contents of which are hereby incorporated by reference in its entirety]. Alternatively, a yeast artificial chromosome (YAC) that encodes a BCRP variant for example, can be used. In a preferred embodiment the transgenic animal is a mouse.

Alternatively, an animal model can be prepared in which expression of the *BCRP* gene is disrupted. Gene expression is disrupted, according to the invention, when no functional protein is expressed. One standard method to evaluate the phenotypic effect of a gene product is to employ knock-out technology to delete a gene as described in U.S. Patent 5,464,764, Issued 11/7/95; and U.S. Patent 5,777,195, Issued July 7, 1998 (both of which are hereby incorporated by reference herein in their entireties.)

In a specific embodiment, PCR can be used to generate a probe encompassing the first 500 basepairs, for example, of the *Bcrp1* cDNA. This probe can be used to screen a commercially available library of bacterial artificial chromosomes (BACs). The BAC

comprising the corresponding *Bcrp1* gene is then used in typical gene targeting methodology.

The knockout mice can be constructed by replacing the ATP-binding cassette of *Bcrp* with Neo^R targeting constructs for example. The resulting *Bcrp* knockout mice can be analyzed for hematopoietic function using standard assays, and for stem cell content using competitive repopulation assays. Hematopoiesis can also be assessed in the embryo and transplant studies can be perform with fetal liver cells. Alternatively, conditional knockouts can be prepared in which the ATP-hydrolysis region is flanked by LoxP sites. Bone marrow cells from the adult mice then can be transduced with a retroviral vector that expresses both Cre recombinase, and a linked marker gene, *e.g.*, green fluorescent protein. Transduced cells are sorted for marker expression. Quatitative repopulating studies can be performed to correlate loss of *Bcrp* expression with defects in stem cell function.

The *BCRP* knockout mice also can be crossed with *mdr1a/1b* knockout mice. *Mrp4* mice also can be crossed either with *Bcrp* -/-, with the *mdr1a/1b* knockout mice or with the *Bcrp* -/-, *mdr1a/1b* triple knockout mice. These mice can be used to determine whether one or more of the particular ABC transporters are required for stem cell function, since the absence of repopulating stem cells in any of the particular knockout mice will indicate that the corresponding ABC transporter(s) is indeed required. In addition, other ABC transporters from sorted SP cells can be expression cloned and studied as described above.

In yet another aspect of the invention a knockin animal is made. A knockin animal is prepared in an analogous manner as a knockout animal except a variant/modified exon or gene is substituted for the exon or gene of interest through homologous recombination, rather than disrupting the gene. A gene-targeting strategy can be used that utilizes a replacement vector containing a particular point mutation and a *neo* gene flanked by loxP sites to construct the mutation in mice. This procedure is known as the Pointlox procedure [Giese *et al. Science* **279**:870-873 (1998)].

The present invention also extends to the preparation of antisense nucleotides and

ribozymes that may be used to interfere with the expression of the *Bcrp* gene. These approaches utilize either an antisense nucleic acid to block translation of a specific mRNA, by masking that mRNA with an antisense nucleic acid or a ribozyme that specifically cleaves the mRNA.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule [*See* Weintraub, *Sci. Amer.* **262**:40-46 (1990); Marcus-Sekura, *Nucl. Acid Res,* **15**: 5749-5763 (1987); Marcus-Sekura *Anal.Biochem.*, **172**:289-295 (1988); Brysch *et al.*, *Cell Mol. Neurobiol.*, **14**:557-568 (1994)]. Preferably, the antisense molecule employed is complementary to a substantial portion of the mRNA. In the cell, the antisense molecule hybridizes to that mRNA, forming a double stranded molecule. The cell does not translate an mRNA in this double-stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Preferably a DNA antisense nucleic acid is employed since such an RNA/DNA duplex is a preferred substrate for RNase H. Oligomers of greater than about fifteen nucleotides and molecules that hybridize to the AUG initiation codon will be particularly efficient. Antisense methods have been used to inhibit the expression of many genes *in vitro* [Marcus-Sekura, *Anal.Biochem.*, **172**:289-295 (1988); Hambor *et al.*, *Proc. Natl. Acad. Sci. USA* **85**:4010-4014 (1988)] and *in situ* [Arima *et al.*, *Antisense Nucl. Acid Drug Dev.* **8**:319-327 (1998); Hou *et al.*,*Antisense Nucl. Acid Drug Dev.* **8**:295-308 (1998)]. The DNA sequences of BCRP included herein may thus be used to prepare antisense molecules against mRNAs encoding the BCRP protein.

BCRP Expression and AML

A significant number of AML blasts can efflux fluorescent dyes, despite the absence of MDR1 and MRP1 expression. A substantial proportion of these cases may be due to the overexpression of BCRP. AML blasts from newly diagnosed pediatric patients can be assayed using flow cytometry after staining with a specific anti-BCRP antibody. In cases where BCRP expression is dysregulated, it can be determined whether the overexpression BCRP is due to mutations in the promoter, hypomethylation of promoter sequences, or due to changes in the transcription factor

environment. The correlation between BCRP expression, the response to induction treatment, and the prognosis can then be made.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. These examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1

EX VIVO EXPANSION OF MURINE HEMATOPOIETIC STEM CELLS TRANSDUCED WITH AN MDR1 RETROVIRAL VECTOR

Introduction

Initial studies were intended to determine whether gene-modified progenitors could be expanded in culture and whether these relatively mature transduced cells would be useful for chemoprotection against myelosuppression in mice receiving antifolate chemotherapy. This progenitor approach previously has been shown effective with vectors expressing methyguanine DNA methyltransferase [MGMT] and protective against 1,3-bis(2-chloroethly)-1-nitrosourea [BCNU] mediated delayed myelosuppression. Unexpectedly, mice receiving bone marrow transduced with an MDR1 retrovirus showed sustained engraftment in non-irradiated recipients. In addition, subsequent quantitation of the stem cell content by competitive repopulation experiments in lethally-irradiated mice indicated a large increase in the repopulation potential with expanded MDR1 marrow. Such results were surprising since there had been numerous observations of negative effects of the expansion of stem cells when alternative gene-modified progenitors had been generated. A myeloproliferative disorder did result in some cases, since a fraction of mice engrafted with MDR1 marrow developed a myeloproliferative disorder characterized by high peripheral white blood cell counts and splenomegaly. This disorder, however, was not found to be absolutely linked to the stem cell expansion described herein.

Methods

Retroviral producer cell lines and vector constructs: The Harvey (Ha)MDR1 and HaDHFRL22Y vectors and ecotropic producer cell lines were generated as described previously [Sorrentino *et al.*, Science **257**: 99-103 (1992); Galipeau *et al.*, *Human Gene Therapy* **8**:1771-1783 (1997), hereby incorporated by reference in their entireties]. The MDR1 protein (encoded by SEQ ID NO:1 and having the amino acid sequence of SEQ ID NO:2) contains a wild-type glycine 185 amino acid. Wild-type MDR1 shows increased resistance to etoposide and decreased resistance to vinca alkaloids when compared with the valine 185 mutant (encoded by SEQ ID NO:3 and having the amino acid sequence of SEQ ID NO:4). The MDR1 cDNA has been previously splice-corrected to allow for optimal levels of active protein expression in transduced cells [Galipeau *et al.*, *Human Gene Therapy* **8**:1771-1783 (1997)]. The DHFRL22Y protein contains a leucine to tyrosine mutation at codon 22 (L22Y) which greatly optimizes resistance to trimetrexate [Spencer *et al.*, *Blood* **87**:2579-2587 (1996)].

Retroviral-mediated bone marrow transduction: Bone marrow cells were flushed from the hind limbs of either C57/Bl6 (C57) or B6.C-H1/BY (HW80) congenic mouse strains (day -4) and prestimulated for 48 hours in Dulbecco's modified essential medium (DMEM; BioWhittaker, Walkersville, MD) supplemented with 15% fetal bovine serum, 100 units/ml penicillin, and 100 ng/ml streptomycin (P/S; Gibco-BRL). Growth factors were also included in the suspension culture at the following concentrations; 20 ng/ml murine IL-3 (Amgen), 50 ng/ml human IL-6 (Amgen), and 50 ng/ml murine SCF (Amgen and R & D Systems) as previously described. Following prestimulation (day -2), cells were co-cultured on irradiated (1500 rads) GP+E86 ecotropic producer cell lines for 48 hours in the presence of the same growth factor combination but also with added 6 mg/ml polybrene (Sigma) to enhance transduction. C57/Bl6 donor mice have a single hemoglobin (Hb) pattern while HW80 have a diffuse Hb pattern when separated on cellulose acetate gels (Helena Laboratories, Beaumont, Texas). These Hb patterns were subsequently utilized for characterization of engraftment.

Ex vivo culture and expansion of myeloid progenitors: Following transduction (day 0), cells were cultured in the presence of the growth factor combination described above. Cells were resuspended at 1 x 10^6 cells/ml every 3-6 days for at least 12 days of expansion. Aliquots of cells were removed for CFU-C analysis at various time points. The percentage of drug-resistant progenitors was calculated by plating cells in methylcellulose (Stem Cell Technologies) in the presence of selective concentrations of drugs. MDR-transduced progenitors were resistant to 50 ng/ml taxol and DHFR-transduced progenitors were resistant to 25 to 50 nM trimetrexate. These concentrations of trimetrexate completely killed non-transduced background cells when plated in thymidine phosphorylase-treated methylcellulose. The percentage of transduced cells was found to remain constant through expansion. Average progenitor transduction efficiencies were: MDR1 TaxolR (40.3 + 10.2%), DHFR TrimetrexateR (39.6 + 17.8%).

Non-irradiated recipient bone marrow transplants: During bone marrow transplant into non-irradiated recipients, mice received 5 daily intravenous injections with either MDR- or DHFR-transduced bone marrow cells (total of 20 - 40 x 10^6 cells for the 5 day period). Later each day mice also received intraperitoneal injection with trimetrexate (130 mg/kg) and the nucleoside transport inhibitor nitrobenzylmercaptopurine riboside phosphate (NBMPR-P; 20 mg/kg). Following this five day treatment course, the presence of donor Hb was monitored in recipient mice beginning at one week and followed for 8 to 14 months. Trimetrexate-glucuronate was received as the base from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI. It was converted to the glucuronate form as described previously [Spencer *et al.*, *Blood* **87**:2579-2587 (1996)].

Competitive repopulation assays: Expanded MDR1 transduced cells were mixed either with the indicated donor hind limbs volume of 12 day expanded DHFRL22Y transduced cells or with freshly harvested marrow. Cells were mixed thoroughly and injected via the tail-vein into lethally-irradiated (925 - 1000 rads) recipient mice. Beginning at 10 weeks post-transplant, Hb patterns were analyzed by electrophoresis on cellulose acetate gels to calculate the relative proportions of single and diffuse

donor hemoglobin in reconstituted mice. The results of these analyses were quantitated by densitometry.

Secondary bone marrow transplants: Bone marrow was harvested from primary recipients from 10 to 24 weeks following transplant and injected into lethally-irradiated secondary recipients. Secondary transplanted mice received at least 5×10^6 bone marrow cells. Hb patterns were monitored in secondary recipients after reconstitution (8 to 10 weeks). Secondary CFU-S were harvested 12 days following injection of $1 - 5 \times 10^4$ cells and DNA was prepared for Southern blot analysis for the presence of the MDR1 transgene.

Southern blot analysis: DNA was prepared as previously described [Sorrentino *et al.*, Science **257**: 99-103 (1992)]. Typically 10 to 20 mg of genomic DNA was restriction digested with either EcoR1 or NheI, and separated on a 1% agarose gel. Gels were blotted overnight onto Hybond N+ nylon membrane (Amersham), UV crosslinked, and hybridized with either MDR1 or hemoglobin-specific [32P]-labeled probes. Blots were washed extensively at 65°C, exposed overnight, and analyzed on a phosphorimager (Molecular Dynamics).

Detection of human p-glycoprotein on mouse erythrocytes: One microliter of murine whole blood containing approximately 6×10^6 erythrocytes was washed in PBS and resuspended in 0.5 ml of Fc-Block (Pharmingen, San Diego, CA) in a final volume of 20 ml of PBS. The red cells were washed and resuspended in PBS with 0.3 mg of unlabeled primary anti-human Pgp monoclonal murine antibody 4E3 (M3523, Dako Corporation, Carpinteria, CA). Cells were next incubated with primary antibody for 45 minutes at room temperature. Cells were then washed and incubated with a phycoerythrin-linked goat anti-mouse IgG antibody (Caltag Laboratories, Burinlgame, CA) for 30 minutes at room temperature. Cells were next washed and analyzed by flow cytometry. Red cells and platelets were identified by the characteristic forward and side scatter distribution. Red cells were gated and analyzed for PE. Leukocytes are located within the erythrocyte gate but these constitute less than 0.1% of all events.

Detection of human p-glycoprotein on mouse leukocytes: Murine whole blood was collected in heparinized tubes and diluted in PBS. Red blood cells were lysed in Gey's solution for 5 minutes on ice. White blood cells were blocked for 15 minutes in PBS/0.1% BSA/10% normal mouse serum followed by staining with a FITC-labeled murine monoclonal anti-human p-glycoprotein antibody (4E3-FITC; Signet Laboratories Inc., Dedham, MA) or with the isotype control. Cells were then analyzed by flow cytometry for FITC-positivity. Gates were drawn on the abnormal population apparent on forward and side scatter profiles in mice with a myeloproliferative disorder.

Assays for replication-competent retrovirus: Genomic DNA was prepared from either cultured producer cell lines, transduced 3T3 cells, or peripheral white blood cells. PCR was performed using primers specific for the 3' end of pol and the 5' end of env regions of the helper virus genome which have been previously described [Scarpa, *Virology* **180**:849 (1991)]. PCR was performed under the following conditions: 94°C, 1.5 minutes denaturation; 55°C, 1.0 minute annealing; 72°C, 1.5 minutes extension; 28 cycles. In addition, marker rescue assays were performed on supernatant from producer cell lines and plasma samples from transplanted mice. A M.dunni/G1Na-transduced cell line was used as the target for marker rescue. Following addition of supernatant, M. dunni/G1Na cells were cultured for at least 2 weeks and supernatant from these cells was assayed at intervals for liberation of infectious retrovirus containing the neo gene. Supernatant were transferred to naive M.dunni cells for 48 hours followed by selection in 0.8 mg/ml G418 (active). No G418R colonies were obtained in multiple experiments at a concentration in which M. dunni/G1Na cells were highly resistant.

Stem cell expansion calculation: 0.005/0.25 + 0.005 = 0.02 predicted engraftment percentage if no stem cell expansion had occurred. Observed engraftment levels = 0.25 to 1.0 indicate an increase in stem cell content of at least 10-fold. It should be noted that engraftment levels were constantly increasing thus any single measurement likely underestimates the true stem cell expansion.

Results

Ex vivo expansion of retrovirally-transduced murine myeloid progenitors was performed as follows. Bone marrow cells were harvested from either C57/Bl6 (C57) or B6.C-H1/BY (HW80) mice (day -4) and transduced by co-culture on retroviral producer cell lines. Producer cells included an MDR1sc producer which expresses the splice-corrected version of the human MDR1 cDNA and a resistance-conferring dihydrofolate reductase (DHFR) mutant referred to as DHFRL22Y. Following transduction (day 0), cells placed into liquid suspension culture for a period of 12 days expanded logarithmically (Fig. 1A). At three day intervals, an aliquot of cells was removed and plated in methylcellulose for assay of the progenitor content. Total progenitor and drug-resistant progenitor levels were calculated and monitored over time. The percentage of progenitors within the total cell population peaked at levels close to 20% between 3 and 6 days following initiation of culture and then gradually declined with time. This transient relative enrichment in progenitors is likely due to death of differentiated cells and expansion of the progenitor pool. The absolute numbers of both drug-resistant and drug-sensitive progenitor populations expanded equally during culture. Thus, the relative percentage of drug-resistant progenitors remained constant throughout the 12 days in culture. Examples of representive expansions are shown for both MDR1 and DHFR-transduced drug-resistant progenitors (Fig. 1B). Typical expansions yielded a 100-fold increase by 2 weeks.

Long-term engraftment of MDR-transduced hematopoietic stem cells was observed in non-irradiated recipient mice. The expansion of cells capable of long-term engraftment in a non-irradiated mouse model was unexpected. MDR- or DHFR-transduced and expanded cells were initially injected into mice with the purpose of testing whether short-term engraftment of drug-resistant progenitors would be protective against antifolate-induced myelosuppression. Recipient mice were treated for 5 days with trimetrexate in combination with the nucleoside transport inhibitor nitrobenzylmercaptopurine riboside phosphate (NBMPR-P). Mice were injected with 12-16 day-expanded cells on each of the 5 days of drug treatment. Following transplant the donor hemoglobin (Hb) levels were monitored in the recipient mice beginning at 1 week and continued through greater than 1 year following injection (Figs. 2A-2B). Donor Hb was present in varying levels in all mice receiving cells as

early as 1 week following injection (the earliest time point examined). However, this engraftment was only transient in mice receiving DHFR- or mock-transduced marrow (0/16; from 2 separate expansion experiments, Fig. 2A). By contrast, 5/12 mice which received MDR-transduced marrow showed long-term engraftment which was stable for more than 6 months post BMT (Fig. 2B), and up to 14 months in the latest time point obtained. Representative Hb electrophoresis profiles for primary engrafted recipients demonstrated the presence of C57 donor Hb at time points 5 to 7 months following injection (Figs. 3A-3B). In addition, secondary recipients from mouse #20 showed a range from 50 - 100% C57 donor Hb 8 weeks after transplant indicating engraftment of the primitive long-term repopulating cells (Fig. 3C). High level expression of P-glycoprotein was seen in donor red blood cells at greater than 10 weeks following transplant in all 4 mice engrafted from experiment #1 (Fig. 3D). The FACS data shows expression in total red blood cells and when corrected for donor chimerism at the same day of analysis the levels were found to range from 80 - 100% positivity. DHFR mouse #1 served as a negative control for P-glycoprotein expression.

Expansion of the MDR-transduced hematopoietic stem cells was quantified by competitive repopulation assay. Thus, to examine whether stem cell expansion was responsible for the high levels of engraftment obtained in the non-irradiated model, a competitive repopulation model was used. MDR- and DHFR-transduced cells were expanded over a 12 day period as described in the Methods, above. Table 1 shows the calculation of the percent hind limbs volume remaining at various time points during the expansion. On day 12, MDR-transduced cells were mixed in an equal volume:volume ratio with expanded day 12 DHFR-transduced cells and injected into lethally-irradiated recipient mice (Figure 4A, right). Also, expanded MDR cells (0.005 C57 donor volumes, Figure 4A, left) or expanded DHFR cells (0.005 C57 donor volumes Figure 4A, middle) were mixed with freshly isolated cells with the opposing Hb pattern (0.25 donor volumes) and injected into lethally-irradiated recipients. Beginning at 10 weeks the Hb patterns in recipient mice were analyzed by Hb electrophoresis (Figure 4C). Interestingly, MDR-expanded marrow completely out competed identically expanded DHFR marrow in repopulation of recipient mice indicating a much higher stem cell content, (lanes 4 -10 of Figure 4C). But most

surprising was the very high level of engraftment of MDR marrow when competed against fresh marrow (lanes 1 and 2 of Figure 4C.). By contrast, when DHFR marrow was competed against fresh marrow it was completely outcompeted (lanes 3 and 4 of Fig. 4C). In addition, over time mice consistently lost their chimerism and approached 100% donor as was seen in the unirradiated model.

TABLE 1

Calculation of Total Cell Expansion and

Percent Hind Limbs Volume Remaining

	HaMDR			HaDHFR		
Day	**Cell Number***	**Volume fraction replated**	**Fraction hind limbs volume**	**Cell Number***	**Volume fraction replated**	**Fraction hind limbs volume**
0	2.15×10^7	1.00	0.86	2.06×10^7	1.00	1.00
3	9.2×10^7	0.22	0.19	6.2×10^7	0.22	0.22
6	6.6×10^7	0.40	0.076	6×10^7	0.52	0.114
9	8×10^7	1.00	0.076	7.9×10^7	1.00	0.114
12	1.6×10^7	---	0.076	8.2×10^7	---	0.114

Note: On days 3 and 6 a fraction of the cells were replated while the rest were discarded. This is reflected in the fraction hind limbs volume remaining. After 12 days of expansion, 0.005 hind limb volumes were injected per mouse along with 0.25 for fresh competed marrow. The fraction at day 0 was determined by the percent of the total volume used following flushing the bone marrow from both fibias and femurs of a single mouse.

* Cell number values are those prior to cell replating

Expanded stem cells are marked with MDR1 proviral DNA. To determine whether the engrafted donor cells were transduced with the MDR1 virus, secondary day 12 CFU-S were analyzed by southern blot (Fig. 5). Genomic DNA from individual CFU-S were digested with EcoR1 and probed with an MDR1 specific probe. A total of 88/88 CFU-S from 7 primary recipients (6 MDR vs. DHFR mice from competitive repopulation expt. #1, and MDR #15 from non-irradiated expt. #2) were shown to be positive by southern blot for the MDR1 provirus giving a band of the expected size

(3464 bp). In addition, a subset of samples were digested with NheI which indicated the presence of the correct size full-length retroviral transcript (8580 bp). These data link the presence of the transgene and expansion of primitive stem cells.

The correlation of the stem cell expansion with period of time in culture was next investigated. To determine the kinetics of stem cell expansion during culture, an additional competitive repopulation experiment was performed. For this experiment, the donor and recipient hemoglobins were switched to eliminate any possibility that engraftment was related to the donor Hb pattern. Following transduction with the MDR1 retrovirus, cells were cultured and aliquots competed at a ratio of 0.02 vol. MDR (HW80)/0.25 vol. fresh (C57) on days 0, 3, 6, and 12. Engraftment of MDR1 marrow was only seen following at least 3 days of expansion post co-culture and engraftment increased with increasing time in culture (Figure 6A). In order to determine whether the high levels of engraftment shown in the erythroid lineage by hemoglobin electrophoresis were also maintained in other lineages, Southern blot analysis was performed on peripheral blood DNA. Blots were treated with the restriction enzyme EcoR1 and probed with a hemoglobin allele-specific probe (Figure 6B). The results shown in Figure 6B demonstrate that the levels of donor hemoglobin seen in the peripheral blood and the levels of lympho-myeloid reconstitution represented in the peripheral blood cell DNA are in full agreement.

In some of the transplanted mice a myeloproliferative disorder was observed providing the first evidence that overexpression of MDR1, and perhaps other ABC transporters, is directly involved in leukemogenesis. Engrafted mice were therefore, analyzed serially to monitor the level of engraftment over time. In some mice, it was noticed that the peripheral white blood cell (WBC) counts began to rise to abnormal levels (Figure 7A). Shown in Figure 7A are 10 representative engrafted mice from the competitive repopulation experiment #2, above. 2/10 of these mice retain normal WBC counts at the present time despite a very large stem cell expansion. In most of the cases the elevation was extremely rapid and could increase by as much as 10-fold within a few days. Then, after a lag phase of 2-6 months after transplant, a number of mice developed marked peripheral blood leukocytosis, with white blood cell counts that ranged between 100,000 and 450,000 cells per l. Analysis of Wright-stained

blood smears (Fig. 7B) showed a relative increase in an abnormal cell population (bottom 2 panels relative to normal mouse in the top panel).

The disease was found to be transplantable into secondary recipients which rapidly developed the same increases in white cell counts. Analysis of the peripheral blood film showed features similar to early stage chronic myelogenous leukemia in about 2/3 of cases, with immature myeloid forms seen without an increase in blast cells (Fig. 7B). Immunophenotyping of these cells showed a high percentage of Gr-1$^+$ and Mac1$^+$ cells. In about 1/3 of the cases, large blast-like cells were present that did not stain with any known lineage markers, giving a picture that resembled acute myelogenous leukemia (Fig. 7B). With both of these phenotypes, massive splenomegaly was invariably seen, with spleen weights ranging from 8-15 times normal size. The disease was found to be transplantable to secondary recipients, confirming that the disorder was occurring at the stem cell level.

The incidence of the myeloproliferative disorder was related both to the length of time following transplant, and to the preceding degree of stem cell expansion. In mice transplanted with freshly transduced cells, the syndrome developed relatively late after transplant and with a relatively low incidence. In contrast, mice transplanted with transduced cells that had been expanded for 12 days developed the syndrome earlier and at a higher incidence at equivalent time points. These results suggest that the robust stem cell amplification achieved during *ex vivo* culture accelerated the development of the myeloproliferative disorder, perhaps by increasing the risk for a second genetic mutation to be acquired during proliferation in the stem cell pool.

Replication-competent retrovirus (RCR) assays were also performed extensively on both cell lines and plasma from mice with the myeloproliferative disorder. A very sensitive PCR assay for helper virus failed to detect the helper genome but was highly positive when using positive control monkey DNA. In addition, marker rescue assays on Mus dunni cells eliminated the possibility of contamination with retroviruses of a wide host range. These data indicate that the stem cell expansion and subsequent myeloproliferative disorder are not due to a contamination of helper virus.

In addition to the elevated WBC count, the number of clonogenic progenitors in the peripheral blood and spleen increased dramatically. Typical progenitor numbers in the blood of a normal animal were 1 - 4/105 cells. Progenitor counts in some mice ranged from 57 to 1290/105 cells. Splenomegaly was also seen in mice with the myeloproliferative disorder. Spleen weights ranged from 483 to 834 mg compared to 106 + 48 mg for normal mice. The progenitor content in the spleen was concomitantly increased from a normal of 3.3-18/105 to 180/105 cells.

On the one hand, the myeloproliferative disorder is not a necessary consequence of the engraftment of the expanded gene-modified hematopoietic stem cells transduced with a nucleic acid encoding MDR1. However, on the other hand, the results disclose herein, indicate that emergence of a single, high copy number stem cell clone with relatively high degrees of MDR1 expression can be an initial step in the development of the myeloproliferative syndrome.

Importantly, despite the abnormal hematologic phenotype, the mice appeared grossly normal and healthy even with the highest white blood cell counts. Analysis of the bone marrow revealed no morphological abnormalities consistent with leukemia. In addition, the mouse karyotype was normal and there were no chromosome translocations present in peripheral blood metaphases from the two representative mice examined. These data are consistent with a prolonged period of abnormal myeloproliferation with transformation to leukemia in only a minority of mice. Peripheral blood cells from several diseased mice were also injected into SCID mice without the development of tumors. Importantly, a percentage of mice have shown large increases in stem cell content and have maintained normal hematologic parameters for as long as 9 to 14 months following transplant. The myeloproliferative syndrome can be dissociated from the hematopoietic stem cell expansion as shown by a significant number of healthy transplanted mice in which there was no evidence of myeloproliferative syndrome.

EXAMPLE 2

ENFORCED P-GLYCOPROTEIN PUMP FUNCTION IN MURINE BONE MARROW CELLS RESULTS IN EXPANSION OF SIDE POPULATION (SP) CELLS *IN VITRO* AND REPOPULATING CELLS *IN VIVO*

Introduction

The human multidrug resistance-1 (MDR1) gene product, P-glycoprotein (P-gp), is well known for its ability to confer drug resistance, however recent evidence suggests that P-gp expression can have more general effects on cellular development. The presence of transport activity in hematopoietic stem cells suggests the possibility that ABC transporters such as MDR1 could have a functional role in stem cell regulation. Further support of this hypothesis is derived from Example 1 above, which disclosed the MDR1 overexpression in murine hematopoietic stem cells. These studies showed that enforced expression of the MDR1 gene, achieved using a retroviral vector, resulted in marked expansion of repopulating stem cells during 12 days of culture in cytokine-containing media [*see also* Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)]. Some mice transplanted with these cells developed a myeloproliferative syndrome phenotypically resembling chronic myelogenous leukemia, demonstrating that dysregulated P-gp expression can adversely affect hematopoietic development.

Materials and Methods

Vector constructs and producer cell lines. The HaMDR1 retroviral vector was constructed using the Harvey (Ha) murine sarcoma virus backbone as has been previously described above [*see* Example 1 above, and *see also*, Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)]. Note that this MDR1 cDNA differs from an earlier vector [Sorrentino *et al.*, *Science*, **257**:99-103 (1992)] in that the sequence for codon 185 has been changed to encode for a glycine residue, and aberrant splicing sites [Sorrentino *et al.*, *Blood*, **86**:491-501 (1995)] have been modified by two point substitutions in the wobble positions of codons 139 and 733 [Galipeau *et al.*, *Hum. Gene Ther.*, **8**:1773-1783 (1997)]. All ecotropic producer cell lines were generated in the GP+E86 packaging cell line [Markowitz *et al.*, *J. Virol.*, **62**:1120-1124 (1988)] using previously described methods [Persons *et al.*, *Blood Cells Mol. Dis.*, **24**:167-182 (1998)], and were shown to be free of replication-competent retrovirus by both PCR

and marker rescue assays. The biological titer of the HaMDR1 ecotropic retrovirus was 2 x 10^5 particles/ml of supernatant as determined by infection of 3T3 cells and quantitation of MDR1 transduction by Rho 123 efflux and by antibody staining (see below). The HaDHFR^{L22Y} vector was used as a control, and expresses a antifolate resistant variant of the human dihydrofolate reductase gene as has been previously described [Spencer *et al.*, *Blood*, **87**:2579-2587 (1996)].

The "pump dead" MDR1 vector: HaMDR1Δ34 utilized a mutant MDR1 cDNA encoding a 34 amino acid deletion in the linker region between the two ATP-binding cassettes [Hrycyna *et al.*, *Biochemistry*, **37**:13660-13673 (1998)]. This mutant cDNA was modified to eliminate potential aberrant mRNA splicing and to encode for glycine at codon 185 as described above, and then inserted into the Harvey murine sarcoma vector backbone. A polyclonal population of ecotropic producer cells was derived by transducing GP+E86 cells with a transient supernatant derived from transfected 293T cells. Vector-transduced producer cells were then isolated by cell sorting for surface P-gp expression as described below. The 4E3 antibody staining procedure was also used to titer the polyclonal HaMDR1Δ34 vector supernatants on 3T3 cells, and showed a titer of 2 X 10^5 particles per ml.

4E3 antibody and Rhodamine 123 staining: Producer cells were analyzed for P-gp expression by staining with a monoclonal mouse anti-human P-glycoprotein antibody (clone 4E3, DAKO, Carpinteria, CA). Adherent cells were trypsinized, and resuspended in 50 ul PBS containing 2% BSA and 0.1% NaN3. 5 ul of the 4E3 antibody was then added, incubated at room temp (RT) for 30 minutes, washed twice with phosphate buffered saline (PBS), and then resuspended in 50 ul PBS containing 2% BSA and 0.1% NaN3. After the primary antibody staining, 5 ul of PE-conjugated, rabbit anti-mouse antibody (DAKO) was added as a secondary stain. The cells were then incubated at RT for 30 minutes, washed twice with PBS, resuspended in PBS for FACS analysis.

Rhodamine 123 (Rho123) staining: Rho123 staining was done by trypsinizing cells, resuspending the cells in DMEM medium containing 10% FCS at a concentration of 1 X 10^6 cells ml, and adding Rho123 (Sigma) at a final concentration of 1 ug/ml. The

cells were then incubated at 37°C for one hour in the dark, washed once with10 mls of PBS, and resuspended in DMEM/10% FCS. The cells were then incubated at 37°C for one hour to allow for efflux, spun down, and then resuspended in 1 ml of PBS for FACS analysis.

Retroviral-mediated gene transfer into murine hematopoietic stem cells: BM cells were harvested from C57BL/6 or B6.Ch-1<b>/By (referred to as "HW80") congenic mouse strains (Jackson Laboratories, Bar Harbor, ME) by standard methods. Following isolation, cells were placed into liquid suspension culture in Dulbecco's Modified Eagle's Medium (DMEM) (BioWhittaker, Walkersville, MD) with 1% penicillin/ streptomycin (Gibco/BRL, Grand Island, NY), 15% fetal bovine serum (FBS; Hyclone, Logan UT), 20 ng/ml murine interleukin (IL)-3 ® & D Systems, Minneapolis, MN), 50 ng/ml human IL-6 (Amgen, Thousand Oaks, CA), and 50 ng/ml murine stem cell factor ® & D Systems). The cells were initially plated at 1 X 10^6 cells/ml in 10 mls of medium. Following pre-stimulation for 48 hours, cells were replated onto confluent monolayers of irradiated ecotropic producer cell lines. The bone marrow cells were plated at the same density used in the pre-stimulation phase and in the same medium with 6 μg/ml polybrene added. Co-culture with producer cells was continued for 48 hours followed by harvest of bone marrow cells. A small sample of bone marrow cells were plated into methylcellulose to score drug-resistant myeloid progenitors. Using the HaDHFR^{L22Y} vector, 68-71% of progenitors were resistant to trimetrexate, and using the HaMDR1 vector, 47-62% of progenitors were resistant to Taxol (n=2 for both vectors), at drug concentrations that killed 100% of control colonies.

Bone marrow expansion cultures: Expansion cultures were initiated immediately after the coculture phase of transduction, which was designated as day 0 of expansion. Non-adherent bone marrow cells were gently removed by pipetting off the medium, followed by washing the producer cells twice with 5 mls of PBS. During these steps, care was taken not to disrupt the producer cell layer. Cells were then centrifuged, media removed, and the cells were replated in suspension culture dishes at a total of 1 X 10^5 cells in 10 mls of medium [Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)]. The

media used for expansion was DMEM that was supplemented with 15% heat inactivated fetal calf serum, and in some experiments, with a commercially available preparation of BSA, insulin, and soluble transferrin (BIT, Stem Cell Technologies, Vancouver, Canada). The media also contained 20 ng/ml of murine IL-3, 50 ng/ml human IL-6, and 50 ng/ml murine SCF. Cells were cultured in non-treated suspension dishes (Corning, Corning, NY) and grown in 5% CO_2 at 37°C in a standard humidified tissue culture incubator. The cells were split on days 3, 6, and 9 and re-seeded at 1 X 10^6 cells/ ml in 10 mls.

Hoechst 33342 SP cell assay: Murine bone marrow cells were collected and resuspended at 1 x 10^6 cells/ml in DMEM plus 10 mM HEPES and 2% FBS. In a water bath, the cells were allowed to equilibrate at 37°C, followed by addition of 5 µg/ml Hoechst 33342 (Fisher Scientific, Pittsburgh, PA) for 90 minutes as previously described [Goodell *et al.*, *J. Exp. Med.*, **183**:1797-1806 (1996)]. Cells were then centrifuged at 4°C and resuspended in ice cold HBSS plus 10 mM HEPES and 2% FBS at 1 X 10^7 cells/ml. For flow cytometric analysis or sorting, a Becton Dickinson FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA) was configured for dual emission wavelength analysis as previously described [Goodell *et al.*, *J. Exp. Med.*, **183**:1797-1806 (1996)]. Cells were gated based on forward and side light scatter to exclude debris. For experiment 2 using the HaMDR1Δ34 vector, propidium iodide staining (2 µg/ml) was utilized to derive a gate excluding dead cells. Cells were analyzed at approximately 5,000 cells/second until data from 1 x 10^6 cells were collected. The SP cell gate was defined based on normal fresh C57BL/6 bone marrow cells.

Analysis of sorted Hoechst 33342 SP cells for stem cell activity: Sorted SP cells were collected in 100 ml of FBS. For limiting dilution analyses, sorted SP cells from C57BL/6 mice were mixed with 2 x 10^5 fresh normal bone marrow cells from congenic HW80 mice to rescue mice from lethal irradiation (1100 rads; ^{137}Cs source). Both hemoglobin electrophoresis [Whitney, *Biochem. Genet.*, **16**:667-672 (1978)] and PCR of peripheral blood leukocytes for MDR1 vector sequences were performed to assay for reconstitution in mice 16 weeks following transplant. The P7 and P8 PCR

primers and conditions used have been previously described [Sorrentino *et al.*, *Blood*, **86**:491-501 (1995)].

Transplants and competitive repopulation assays: Donor bone marrow cells were mixed at the indicated ratios and injected into the tail vein of HW80 recipient mice that had been lethally irradiated with 1100 cGy using a ^{137}Cs γ-irradiator. Peripheral blood was obtained by retro-orbital bleeding in anesthetized mice at varying time points after reconstitution and analyzed by hemoglobin electrophoresis or DNA PCR. Hemoglobin electrophoresis was performed on cellulose acetate plates as previously described [Whitney, *Biochem. Genet.*, **16**:667-672 (1978)] using a commercially available kit (Helena Laboratories, Beaumont, TX). For PCR, genomic DNA was isolated from the circulating leukocytes present in 70 μl of blood using the InstaGene Genomic DNA kit (BioRad, Hercules, CA), and resuspended in 20 μl of water. One μl of the DNA solution was amplified using a commercially available kit (Qiagen Inc., Valencia, CA) and the following parameters (35 cycles, 94°C X 1', 60°C X 1', 72°C X 1'. The PCR primers used to amplify fragments from the HaMDR1and HaMDR1Δ34 vectors were as follows:

5' CCACGTCAGCCTTGGACACA 3' (SEQ ID NO:15)

5' GCCGCTTGGTGAGGATCTCT 3' (SEQ ID NO:16)

Results

The goal of the present example study was to further explore the effect of MDR1 gene expression on stem cell development. Considering the link between Side Population (SP) stem cells and transporter function, it was initially asked whether MDR1-mediated stem cell expansion was associated with an increase in SP stem cells in expansion cultures. It was also determined whether stem cell expansion was limited to *ex vivo* culture conditions. For instance, the mechanism of stem cell expansion could be due to efflux of some media component that had negative effects on stem cell proliferation. An alternative and more interesting possibility is that MDR1 gene expression could be acting at a more global level that is independent of *ex vivo* culture conditions. To distinguish between these possibilities, it was determined as to whether freshly transduced stem cells would have a direct proliferative advantage *in*

vivo after transplantation. In addition, experiments were performed to determine whether these stem cell effects required the efflux-pump activity of P-gp, or whether the effects could be due to other properties of the experimental system. A vector encoding an expressed but functionally dead P-gp was tested in both the SP cell expansion assay and *in vivo* competitive repopulation assays. Altogether, these studies provide further evidence that enforced ABC transporter function can alter the proliferative and developmental fate of hematopoietic stem cells.

Expansion of MDR1-transduced SP cells during ex vivo culture: To determine whether enforced P-gp expression would result in an increase in SP cell numbers during *ex vivo* expansion cultures, murine BM cells were transduced with the HaMDR1 retroviral vector [Example 1, above; Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)] and then cultured for 12 additional days in media containing fetal calf serum, IL-3, IL-6, and SCF. As a control, cells were transduced with a vector expressing a human dihydrofolate reductase gene (HaDHFR^{L22Y}) within the same vector backbone [Spencer *et al.*, *Blood*, **87**:2579-2587 (1996)]. SP cells were quantitated by Hoechst 33342 staining and flow cytometry after 0, 6, or 12 days of culture (Fig. 8). In populations of cells transduced with the HaDHFR^{L22Y} vector, a loss of cells in the lower part of the SP tail, corresponding to stem cells with long-term repopulating activity [Goodell *et al.*, *Nat. Med.*, **3**:1337-1345 (1997)], had already occurred immediately following the transduction procedure (day 0). After an additional 6 days of expansion, SP cells were no longer detectable in these control cultures. In contrast, cultures of cells that were transduced with the HaMDR1 vector showed preservation of SP cell numbers at early time points, with an increase in the absolute number of SP cells averaging 187-fold (n = 3, standard error 161, range 38-380 fold) after 12 days of *ex vivo* culture. These data show that while SP cells were lost over time in extended bone marrow cultures, enforced expression of the MDR1 gene resulted in a large amplification of SP cells over a 12-day time period.

Repopulating activity in 12 day-expanded, sorted SP cells. To determine if repopulating cells were present and enriched within the expanded SP population, limiting dilution transplant experiments were performed in irradiated mice using sorted SP cells. Control experiments with normal, fresh BM cells showed reconstitution with SP cell doses as low as 250 cells, consistent with previously

reported values [Goodell *et al.*, *J. Exp. Med.*, **183**:1797-1806 (1996)]. The repopulating cell frequency in the MDR1-transduced SP cell population was determined by transducing C5BL/6 BM cells with the HaMDR1 vector, expanding transduced cells for 12 days, and then sorting for SP cells by flow cytometry. These sorted SP cells were injected into lethally-irradiated recipient mice along with 2×10^5 fresh bone marrow cells (HW80 background), the latter used to confer radioprotection to the mice. Reconstitution analysis was done 16 weeks after transplant using a PCR assay to detect HaMDR1 vector sequences in total peripheral blood leukocyte DNA. Reconstitution was also studied using hemoglobin electrophoresis to determine the relative erythroid contributions arising from sorted SP cells.

The PCR assay showed repopulation of recipient mice with SP cell doses as low as 500 cells, and vector-marked cells were present in the majority of mice receiving between 1500-9400 expanded SP cells (Fig. 9A). In most of these cases, the hemoglobin electrophoresis also showed contributions from the expanded SP cells derived from the C57BL/6 background, however the percent contribution was very low in the majority of mice (Fig. 9B). These data suggest that the expanded SP cell population may be somewhat enriched for repopulating cells relative to whole bone marrow, but that the frequency of repopulating cells in the expanded SP population is significantly less than that in fresh SP cells from normal bone marrow. The low degrees of chimerism for the expanded SP cells could reflect a limited repopulation capacity of the expanded cells, or simply a greater number of stem cells in the fresh HW80 graft used to provide radioprotection. This relative decrease in the concentration of repopulating cells in the expanded SP population suggests that a significant proportion of the expansion is due to a phenotypic change in the cells without a corresponding change in repopulation potential. This interpretation is consistent with previous results showing that the absolute numbers of functionally-defined repopulating cells increased 12-30 fold in MDR1-transduced whole bone cultures [Example 1 above; Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)], while in this study, the absolute number of SP cells increased about 180 fold during the same culture period.

Expansion of MDR1-transduced stem cells in vivo. To determine whether the MDR1 vector was having a direct effect on stem cell proliferation, or whether the effect was specific to *ex vivo* expansion cultures, it was determined as to whether MDR1-mediated stem cell expansion would also occur directly *in vivo*. BM cells were transduced with either the HaMDR1 or the HaDHFR^{L22Y} vector, and these grafts were mixed immediately after transduction for competitive repopulation experiments. C57BL/6 BM cells were transduced with the HaMDR1 vector, and HW80 BM cells were transduced with the HaDHFR^{L22Y} vector. After transduction, equal donor volumes from each graft were mixed and transplanted into lethally irradiated recipient mice. Mice received between 2.3- 5.1 X 10^6 HaDHFR^{L22Y}-transduced cells, and between 1.8- 2.5 X 10^6 HaMDR1-transduced cells. In one experiment, the recipient mice were divided into two cohorts. One group of mice received two 5-day courses of granulocyte colony-stimulating factor (G-CSF) /stem cell factor (SCF) growth factor treatment on weeks 5 and 8 following transplant as has been described [Bodine *et al.*, *Blood*, **88**:89-97 (1996)], and one group of mice was left untreated. Because treatment with these growth factors is known to cause a significant increase in stem cell content *in vivo* [Bodine *et al.*, *Blood*, **88**:89-97 (1996)], the treated mice might show an earlier outgrowth of the MDR1-transduced graft.

At 7 weeks following transplant, hemoglobin reconstitution patterns uniformly reflected the input ratio of the grafts, with a roughly 50/50 mix of the two hemoglobin patterns (Fig. 10A). At later time points, mice from both groups showed a gradual increase in the proportion of erythroid cells derived from the MDR1-transduced graft. At 14 weeks after transplant, the cytokine-treated group showed increased proportions of cells from the MDR1-transduced graft relative to the untreated group, but this did not reach statistical significance ($p = 0.094$). At 24 weeks this difference was no longer apparent, and the MDR1-transduced BM graft had completely overtaken the control graft in most cases. Data from two independent experiments are graphically shown in Figs. 10B-10C. The majority of mice showed a large and progressive shift towards MDR1-transduced donor cell engraftment over time. These experiments demonstrate that the capacity of MDR1 vectors to expand stem cells is not limited to the 12-day culture protocol.

Clonality and copy number in transduced stem cell clones. It was next determined if the outgrowth of MDR1-transduced cells *in vivo* was due to polyclonal stem cell expansion. Six of the primary transplant recipients from expt.#1 were killed 20 to 26 weeks following transplant and the BM cells were injected into irradiated secondary recipients. Day 12 CFU-S colonies were then isolated and DNA was analyzed by Southern blotting for MDR1-vector integration sites. Figure 11 shows two representative Southern blots showing the range of clonality observed in these mice. Mouse #12 showed 4 unique integration patterns within 7 individual CFU-S, indicating that hematopoiesis was polyclonal at a time when MDR1-transduced stem cells had expanded and outcompeted the control graft. It was noted that there was an increased number of clones with relatively high vector copy numbers (12.1, 0.2, 0.4, 0.5, and 0 .7) versus low copy numbers (12.3 and 0 .6). Mouse #10 showed oligoclonal hematopoiesis where all clones but one (10.11) show a highly uniform banding pattern with very high copy number (18 integrants). Some stem cell clones in mouse #10 showed unique integrations (arrows) in addition to the common integration sites. This is most likely due to self-renewing stem cell divisions that occurred during the 48-hour transduction period. Of the 6 primary mice that were analyzed, 3 showed polyclonal stem cell patterns, 1 was oligoclonal, and 2 showed a monoclonal pattern. The increased proportion of clones with relatively high copy numbers may reflect a proliferative advantage for stem cell clones with relatively high levels of P-gp expression.

The development of a functionally inactivated MDR1 vector. It was next determined as to whether the expansion of SP cells and repopulating cells required the efflux-pump function of P-gp. A vector was constructed (HaMDR1Δ34) that was otherwise identical to HaMDR1 except for a coding region deletion resulting in loss of 34 amino acids in the linker region between the two ATP hydrolysis sites. As originally described [Hrycyna *et al.*, *Biochemistry*, **37**:13660-13673 (1998)], this mutant MDR1 cDNA expresses P-gp on the cell surface at relatively normal levels, but the mutant protein cannot act as an efflux pump. As expected, producer cells expressing the HaMDR1Δ34 vector had relatively high levels of P-gp expressed on the cell surface as detected by flow cytometry using the monoclonal antibody 4E3, but lacked the ability to efflux the P-gp substrate Rho 123 (Figs. 12A-12D). The ability

of the HaMDR1Δ34 vector to transduce early bone marrow cells was compared with the HaMDR1 vector by evaluating the transduction frequency of the two vectors in primary CFU-S. This analysis showed that the HaMDR1Δ34 vector was highly efficient at transducing CFU-S, and had a titer at least equivalent to the wild type HaMDR1 vector (Figs. 13A-13B).

The requirement of P-gp pump function for SP cell expansion in vitro. In two separate experiments, bone marrow cells were transduced with the HaMDR1Δ34 vector, expanded in culture, and analyzed at various time points for changes in the number of SP cells. The first experiment was analyzed after 7 days of culture, and showed no SP cell expansion in cultures transduced with the HaMDR1Δ34 vector, while there was a marked expansion of SP cells using the HaMDR1 vector (compare Figs. 14A and 14B). A second experiment showed a complete lack of SP cells in cultures transduced with the HaMDR1Δ34 vector and expanded for 13 days (compare Figs. 14C and 14D). These experiments demonstrate that P-gp pump function was required for expansion of SP cells in culture.

The requirement of pump function for expansion of repopulating cells. The HaMDR1Δ34 vector was next evaluated in competitive repopulation experiments to determine if P-gp pump function was required for expansion of repopulating cells. Bone marrow from C57BL/6 mice was transduced with either the HaMDR1Δ34 or HaMDR1 vector, and then expanded in culture for 12 days. In one experiment, the expanded cells were competed against fresh bone marrow cells from HW80 mice. The input ratio used was 0.02 femur volumes of transduced, expanded marrow to 0.25 femur volumes of fresh HW80 marrow. Sixteen weeks after transplant, the recipient mice were analyzed by hemoglobin electrophoresis. All 6 mice transplanted with HaMDR1 transduced cells showed significant contributions from the transduced graft (Fig. 15A), despite the low input ratio of transduced cells. In contrast, there was no detectable donor contribution from the HaMDR1Δ34 transduced graft in any of the six mice that received HaMDR1Δ34-transduced and expanded cells along with fresh bone marrow cells.

In addition, both transduced grafts were directly competed by transfusing equal volumes of each graft into irradiated recipients, and then using PCR to evaluate the relative contributions to engraftment in peripheral blood leukocytes. PCR primers were developed that flanked the 34 amino acid deletion, and therefore could co-amplify products of two different sizes from the HaMDR1 and HaMDR1Δ34 vectors (692 and 590 bp respectively). In 9 mice from two independent experiments, there was no detectable contribution from the HaMDR1Δ34 vector, while HaMDR1-transduced cells were readily detected (Fig 15B). The presence of the HaMDR1Δ34-amplification product was easily detected in mice transplanted solely with unexpanded, transduced cells, ruling out a lack of stem cell transduction with HaMDR1Δ34 as the explanation for the competition result.

Myeloproliferative disorder in transplanted mice. Recipients of *ex vivo* expanded HaMDR1-transduced BM cells developed a myeloproliferative disease [Example 1 above; Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)]. In this study, the peripheral white blood cell counts of transplanted mice were analyzed at varying times following transplantation, looking for the characteristic leukocytosis that is associated with the myeloproliferative syndrome. In the two groups of mice transplanted with freshly transduced HaMDR1-transduced cells, 3/13 and 1/18 showed leukocyte counts greater than 20,000 cells/ul at 24 and 26 weeks post-transplant respectively. In contrast, in Example 1, above [*see also* Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)] which used cells expanded for 12 days *in vitro*, leukocytosis was observed in 21/24 and 7/7 mice at 25 and 27 weeks respectively. The 4 mice transplanted exclusively with HaMDR1Δ34-transduced bone marrow cells have been followed for 15 weeks, and none have shown leukocytosis or any other abnormalities.

Discussion

Although it is well known that the MDR1 gene is expressed in primitive human hematopoietic cells [Chaudhary and Roninson, *Cell*, **66**:85-94 (1991)], the functional importance of MDR1 gene expression has not been defined. There are several lines of evidence suggesting that expression of the MDR1 gene, or perhaps other ABC transporters, has an important functional role in stem cells. Repopulating stem cells from a variety of species can be purified based on their ability to exclude fluorescent

dyes, a property at least partially attributable to transporter expression. This conservation of transporter expression in stem cells is consistent with an important functional effect. A more direct line of evidence comes from Example 1, above which shows that enforced expression of the MDR1 gene resulted in marked amplification of murine stem cells over a 12-day culture period [*see also* Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)]. The data presented here confirm and extend that disclosed in Example 1 above, and provide further support for the concept that MDR1 transporter function can influence the replicative behavior of stem cells.

An intriguing link between MDR1 expression and normal stem cell function has been the identification of the SP stem cell phenotype. SP stem cells are found in the bone marrow from multiple mammalian species and express low to undetectable levels of the CD34 antigen [Goodell *et al.*, *Nat. Med.*, **3**:1337-1345 (1997)], indicating that they may contain the primitive CD34-negative stem cells that have been recently described in xenograft transplantation experiments [Bhatia *et al.*, *Nat. Med.*, **4**:1038-1045 (1998) and Zanjani *et al.*, *Exp. Hematol.*, **26**:353-360 (1998)]. Because the SP phenotype can be abrogated with verapamil, a known inhibitor of P-gp, it has been suggested that the SP phenotype may be due to expression of the MDR1 gene, or perhaps another ABC transporter [Goodell *et al.*, *J. Exp. Med.*, **183**:1797-1806 (1996)]. Results from experiments disclose herein demonstrate that enforced MDR1 expression resulted in a large amplification of SP stem cells in culture. These data strengthen the functional link between the SP stem cell phenotype and MDR1 gene expression. One possible mechanism for the MDR1-mediated expansion of stem cells is through a reduction of apoptosis in the cultures. CD34+ hematopoietic cells normally undergo apoptosis during *ex vivo* expansion cultures [Traycoff *et al.*, *Exp. Hematol.*, **26**:53-62 (1998)]. It has recently been shown that P-gp can inhibit apoptosis in cultured hematopoietic cells [Johnstone *et al.*, *Blood*, **93**:1075-1085 (1999)], as well as in other cellular systems [Smyth *et al.*, *Proc.Natl.Acad.Sci.USA*, **95**:7024-7029 (1998); Robinson *et al.*, *Biochemistry*, **36**:11169-11178 (1997); and Gruol and Bourgeois, *Biochem. Cell Biol.*, **72**:561-571 (1994)].

In contrast, when DHFR-transduced control cells were cultured, SP stem cells were progressively lost over time. This loss of SP cells correlates with the known loss of

repopulating stem cells with extended culture periods [Bhatia *et al.*, *J. Exp. Med.*, **186**:619-624 (1997); Tisdale *et al.*, *Blood*, **92**:1131-1141 (1998); and Shimizu *et al.*, *Blood*, **91**:3688-3692 (1998)]. Deleterious effects on SP stem cells were even noted in control cultures immediately after the 4-day transduction period. The relative loss of long term repopulating stem cells during transduction with the DHFR control vector may in part explain the difficulty in obtaining durable gene marking in large animal models. Culture conditions have recently been identified that minimize the loss and differentiation of repopulating stem cells [Bhatia *et al.*, *J. Exp. Med.*, **186**:619-624 (1997) and Glimm and Eaves, *Blood*, **94**:2161-2168 (1999)], and it may be that these methods result in a relative preservation of SP stem cells.

To determine if MDR1-mediated stem cell amplification was restricted to the setting of *ex vivo* expansion cultures, it was determined whether freshly transduced cells would have a direct competitive advantage *in vivo* after transplantation. These experiments showed a large selective advantage for the HaMDR1-transduced cells that progressively emerged in transplanted mice over a 6 month time frame. Although it is theoretically possible that some of the observed stem cell expansion may have occurred during the 2 day transduction period, previous results showed very little amplification of repopulating cells at day 0 [Example 1 above; Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)]. Furthermore, the slow emergence of the MDR1-transduced graft over 24 weeks is most consistent with a direct proliferative advantage *in vivo*. These data indicate that MDR1 expression is modulating an endogenous stem cell substrate *in vivo*, rather than simply extruding some component present within the *ex vivo* culture media. In the secondary CFU-S experiments, most stem cell clones were observed to have relatively high copy numbers of the MDR1 vector (8-18 copies). The degree of amplification seen in a given stem cell clone may be directly related to the level of expression of P-gp, consistent with the correlation between copy number and expansion.

Prior to this study, one important mechanistic question was whether the pump function of P-gp was necessary for stem cell expansion. If the pump function were not required, amplification would be due to direct membrane effects from P-gp, or due to some artifacts of the retroviral transduction system. Using a vector that expresses a

functionally inactive P-gp demonstrates that pump function is required for both expansion of SP cells *in vitro*, and expansion of repopulating cells *in vivo*. These findings show that the observed stem cell effects are due to redistribution of a critical substrate within stem cells.

Some mice transplanted with MDR1-transduced cells developed a myeloproliferative syndrome characterized by high white blood cell counts, immature myeloid cells in the peripheral circulation, and splenomegaly; resulting in a syndrome that phenotypically resembles chronic myelogenous leukemia. This syndrome occurred with a decreased incidence and a longer latency period compared to historical controls [Example 1 above; Bunting *et al.*, *Blood*, **92**:2269-2279 (1998)] that were transplanted with *ex vivo* expanded, MDR1-transduced cells. This comparison indicates that the rate of development of the syndrome is related to the prior degree of stem cell expansion, perhaps by increasing the probability of a second genetic event required for transformation. In earlier studies of MDR1 transfer into murine stem cells [Sorrentino *et al.*, *Science*, **257**:99-103 (1992); Podda *et al.* , *Proc Natl Acad Sci U S A* **89**:9676-9680 (1992); Hanania *et al.*, *Gene Ther.*, **2**:279-284(1995)], the development of a myeloproliferative syndrome was not reported. One possible explanation for this discrepancy is differences in the vectors used in the earlier studies. The vector used in our stem cell expansion experiments has been modified to reduce cryptic mRNA splicing within the MDR1 coding sequence [Sorrentino *et al.*, *Blood*, **86**:491-501 (1995); Galipeau *et al.*, *Hum. Gene Ther.*, **8**:1773-1783 (1997)], and expresses greater amounts of P-gp than the vectors used in the previous studies.

The relationship between MDR1 gene expression and the occurrence of a myeloproliferative disorder in the present model system raises the possibility that dysregulated P-gp expression may contribute to the development of leukemia. In patients with acute myelogenous leukemia, between 35 and 70% of cases demonstrate P-gp expression in the pretreatment leukemic blasts, and P-gp expression is a strongly negative prognostic factor [Leith *et al.*, *Blood* **94**:1086-1099 (1999)]. In at least some instances increased P-gp expression in blast cells is due to hypomethylation of sequences in the MDR1 promoter [Nakayama *et al. Blood* **92**:4296-4307(1998)]. In chronic myelogenous leukemia, about 60% of patients show MDR1 expression in

leukemic cells from the bone marrow [Giles *et al. Cancer*, **86**:805-813 (1999)]. While the occurrence of MDR1-negative cases could argue against a necessary role for dysregulated transporter expression in leukemogenesis, it should be noted that high levels of expression of other transporter family members have been identified in a significant number of those cases [Leith *et al.*, *Blood*, **94**:1086-1099 (1999) and Michieli *et al.*, *Br. J. Haematol*, **104**:328-335 (1999)].

EXAMPLE 3

BCRP EXPRESSION CAN BE USED AS A MARKER FOR PURIFICATION OF STEM CELLS

Introduction

Hematopoietic stem cells (HSCs) can be identified by staining with fluorescent dyes such as Rhodamine (Rho) 123 [Orlic *et al.*, *Blood* **82**:762-770 (1993); Fleming *et al.*, *J.Cell Biol.* **122**:897-902 (1993); Spangrude and Johnson, *Proc.Natl.Acad.Sci.USA* **87**:7433-7437 (1990); Zijlmans *et al.*, *Proc Natl Acad Sci USA* **92**:8901-8905 (1995)] and Hoechst 33342 [McAlister *et al.*, *Blood* **75**:1240-1246 (1990); Leemhuis *et al.*, *Exp.Hematol.* **24**:1215-1224 (1996); Wolf *et al.*, *Exp.Hematol.* **21**:614-622 (1993)]. The most primitive HSCs are characterized by low degrees of fluorescence after staining with these dyes, a property ascribed to both their capacity for dye efflux, and to relatively low degrees of mitochondrial staining [Kim *et al.*, *Blood* **91**:4106-4117 (1998)]. A related method for stem cell identification has been based on Hoechst dye-staining of whole bone marrow cells, followed by dual emission wavelength analysis by flow cytometry. This technique identifies a small fraction of side population (SP) cells that are highly enriched for repopulating activity [Goodell *et al.*, *J.Exp.Med.* **183**:1797-1806 (1996)]. The SP phenotype identifies HSCs in a number of mammalian species [Goodell *et al.*, *Nat.Med.* **3**:1337-1345 (1997)], and can be blocked by drugs which inhibit cellular dye efflux mechanisms. It has been thought that the efflux activity responsible for the SP phenotype may be due to expression of P-glycoproteins (P-gps), the products of the mammalian multidrug resistance genes (MDR1 in humans and *mdr1a* and *1b* in mice). This possibility is suggested by the facts that: (i) Rho123 and Hoechst 33342 are substrates for P-gp,

(ii) primitive human hematopoietic cells express high levels of P-gp [Chaudhary and Roninson, *Cell* **66**:85-94 (1991)], and (iii) the phenotype of SP cells can be blocked by verapamil, a competitive inhibitor of P-gp [Goodell *et al.*, *J.Exp.Med.* **183**:1797-1806 (1996)]. More recent evidence shows that the muscle contains reconstituting cells that can be identified by the SP phenotype [Gussoni *et al.*, *Nature* **401**:390-394 (1999); Jackson *et al.*, *Proc.Natl.Acad.Sci.U.S.A* **96**:14482-14486 (1999), *see* comments] suggesting that expression of ABC transporters may be a general stem cell property. Indeed whatever their exact function, it appears that expression of ABC transporters has been evolutionarily conserved in stem cells. The conservation of transporter expression in a wide variety of stem cells is consistent with an important functional role in stem cells.

Although many of the genes encoding ABC transporters were first identified based on their ability to confer drug resistance in tumor cells, it has recently become apparent that they can exert more general effects on cellular function. For example, MDR1 gene expression has been shown to inhibit caspase-dependent apoptosis in a variety of cells [Smyth *et al.*, *Proc.Natl.Acad.Sci.USA* **95**:7024-7029 (1998)] including hematopoietic cells [Johnstone *et al.*, *Blood* **93**:1075-1085 (1999)]. P-gps can also function as lipid translocases by redistributing membrane phospholipids from the inner to outer leaflet of the cell membrane [van Helvoort *et al.*, *Cell* **87**:507-517 (1996)].

Direct evidence that ABC transporters can have a functional effect in HSCs comes from studies of MDR1 gene transfer in mice in Example 1 above. When murine bone marrow cells were transduced with an MDR1-expressing retroviral vector, dramatic expansion of repopulating stem cells was noted during a 12-day culture period [Example 1 above: Bunting *et al.*, *Blood* **92**:2269-2279 (1998)]. In contrast, repopulating activity was lost over time in control cultures [Example 2 above]. These results demonstrate that enforced expression of MDR1 results in stem cell self-renewal and expansion during extended culture periods. This expansion of repopulating cells was associated with a parallel increase in SP cells, while SP cells were lost over time in control cultures. These results directly link ABC transporter expression, or at least MDR1 expression, with the SP stem cell phenotype. One

possible mechanism for the stem cell expansion was that P-gp expression could result in the efflux of toxic media components from HSCs during the *ex vivo* culture period. This possibility was ruled out by the observation that MDR1-transduced stem cells had a direct proliferative advantage *in vivo* [Example 2 above]. When transduced bone marrow cells were directly transplanted in irradiated mice, without an *ex vivo* expansion phase, there was a progressive outgrowth of MDR1-transduced cells relative to a control graft. These results show that MDR1 expression was conferring a more general effect on stem cell division, and not simply acting through a detoxification mechanism specific to *ex vivo* culture. Experiments with a mutant P-gp construct demonstrated that HSC expansion required the molecular pump function of P-gp, suggesting that the mechanism of expansion involved modulation of some endogenous molecular substrate within HSCs [Example 2 above]. Collectively, these studies show that MDR1 gene expression can promote HSC self-renewal and amplification. Heretofore, it was not known if this property is unique to the MDR1 gene, or whether other ABC transporters can exert a similar function.

Clinical evidence also shows an association between dysregulated ABC transporter expression and human leukemia. In chronic myelogenous leukemia, about 60% of chronic phase patients exhibit P-gp expression in leukemic cells from the bone marrow [Giles *et al.*, *Cancer* **86**:805-813 (1999)]. In patients with acute myelogenous leukemia (AML), between 35 and 70% of cases demonstrate P-gp expression in leukemic blasts at diagnosis, and P-gp expression was a strongly negative prognostic factor [Leith *et al.*, *Blood* **94**:1086-1099 (1999)]. In contrast, normal late myeloid cells in humans do not express P-gp [Drach et al., *Blood* **80**:2729-2734 (1992), *see* comments]. In some cases, increased P-gp expression in blast cells was due to hypomethylation of sequences in the MDR1 promoter [Nakayama *et al.*, *Blood* **92**:4296-4307 (1998)]. Expression of other ABC transporters can occur in human AML, as has been documented for the multidrug resistance protein (MRP1), and the lung resistance protein (LRP) [Leith *et al.*, *Blood* **94**:1086-1099 (1999); Michieli *et al.*, *Br.J.Haematol.* **104**:328-335 (1999)]. Importantly, a significant number of AML cases showed inhibitable dye efflux activity that was not associated with MDR1, MRP1, or LRP [Michieli *et al.*, *Br.J.Haematol.* **104**:328-335 (1999); Leith *et al.*, *Blood* **86**:2329-2342 (1995)].

A powerful way to define the normal function of ABC transporters in HSCs is by murine gene disruption experiments. Mice have two closely linked MDR1-like genes that are designated *mdr1a* and *mdr1b*. Both of these genes have been disrupted in single ES cells by a double knockout strategy, and the resulting mice have normal hematologic parameters [Schinkel *et al.*, *Proc Natl Acad Sci USA* **94**:4028-4033 (1997)]. Similarly, the multidrug resistance protein 1 (*mrp1*) gene has been knocked out with no apparent effect on hematopoiesis [Lorico *et al.*, *Cancer Res.* **57**:5238-5242 (1997)]. These findings indicate that expression of these particular ABC transporters is not necessary for HSC function, but do not rule out the possibility that other transporters are providing a critical redundant function in HSCs.

Results

The regulated expression of endogenous P-gps in HSCs appears to be important in facilitating the self-renewal divisions that maintain the stem cell compartment over time (*see* Examples 1 and 2 above) and indeed, one or more naturally occurring endogenous ABC transporters apparently plays a critical functional role in stem cell homeostasis. This premise is consistent with two observations: (i) HSCs universally express dye-effluxing transporters; and (ii) enforced expression of MDR1 leads to stem cell amplification and myeloproliferation (*see* Example 1 above).

As disclosed herein, an alternate ABC transporter(s) is expressed in SP stem cells derived from the *mdr1a/1b* knockout mouse. The analyses of mRNA from sorted SP stem cells have identified several newly cloned transporters that are expressed in SP HSCs, that may possibly have a role in the self-renewal process of HSCs. The most highly expressed is the *Bcrp1/Mxr/Abcp* gene product, BCRP. Importantly, there is no detectable expression of BCRP in peripheral blood leukocytes, spleen, or thymus at the level of Northern blot analysis, while small but detectable amounts of BCRP mRNA were expressed in human fetal liver. BCRP-transfected cells effluxed Rho 123 by an ATP-dependent mechanism, which is consistent with it having a role in dye efflux within stem cells [Doyle *et al.*, *Proc.Natl.Acad.Sci.U.S.A* **95**:15665-15670 (1998): published erratum appears in *Proc Natl Acad Sci U S A*; **96**(5):2569 (1999)].

An ABC transporter other than P-gp is expressed in murine SP HSCs: Quantitative repopulation assays were performed using commercially available *mdr1a/ab* --/-- mice as donors because it is well known that severe quantitative stem cell abnormalities can coexist with relatively normal peripheral blood counts such as in W/W_v mice. Normal numbers of repopulating cells were found to be present in the bone marrow. Bone marrow cells were then analyzed for the content of SP cells after staining with Hoechst dye. To confirm that the knockout mice had the expected phenotype, it was verified that the capacity for Rho 123 efflux had been lost in peripheral blood leukocytes (Fig. 16A-16D) as has been previously described [Schinkel *et al.*, *Proc Natl Acad Sci USA* **94**:4028-4033 (1997)]. Despite this loss of P-gp related transporter function, SP cells were present in normal numbers in the bone marrow when compared to wild type mice of the same strain (Figs. 16A-16B). This indicates that another ABC transporter is likely being expressed, potentially compensating for the loss of P-gp function.

To further test this possibility, biochemical studies were performed on knockout bone marrow cells using known inhibitors of ABC transporter efflux function. Cells were treated with either verapamil or 2-deoxyglucose. Verapamil is a competitive inhibitor of several known ABC transporters including MDR1, whereas 2-deoxyglucose is an inhibitor of ATP synthesis that depletes cellular ATP levels required for ABC transporter function. Treatment with either of these compounds before and during Hoechst 33342 staining eliminated phenotypically identifiable SP cells (Fig. 17). These results conclusively demonstrate that another ABC transporter(s) is being expressed in SP cells from the bone marrow of *mdr1a/1b* knockout mice and is (are) responsible for the SP phenotype.

Identification of Bcrp as an expressed ABC transporter in hematopoietic stem cells: An RT-PCR assay was developed to detect mRNA expression of other known ABC transporters in murine bone marrow SP cells. Based on the published human sequences for MRP1 [Cole *et al.*, *Science* **258**:1650-1654 (1992), *see* comments], MRPs 2, 3, 4 [Kool *et al.*, *Cancer Res.* **57**:3537-3547 (1997)], and BCRP [Doyle *et al.*, *Proc.Natl.Acad.Sci.U.S.A* **95**:15665-15670 (1998):published erratum appears in *Proc Natl Acad Sci U S A*; **96**(5):2569 (1999)] homologous sequences from the

murine EST database were identified to design PCR primers for cDNA amplification. Multiple primer sets were tested using mouse liver cDNA as a template, and primer sets were chosen that gave specific bands of the expected size. Using FACS, bone marrow SP cells were sorted from both normal mice and the *mdr1a/1b* knockout mouse. Total cellular RNA was prepared from 50,000 and 100,000 purified SP cells, and then used for RT-PCR analysis. These experiments showed that the *Bcrp1* mRNA was the most highly expressed of all the transporters studied (Fig. 18). Moderate expression levels were observed for *mrp4* and *mrp1*, while *mrp3* was expressed at very low levels, and no detectable expression of *mrp2* was observed. The low levels of expression of *mrp1* in the liver correlated with previously described low levels of expression of MRP1 in human liver [Kool *et al.*, *Cancer Res.* **57**:3537-3547 (1997)]. Virtually identical results were obtained using sorted SP cells from normal mice.

It is important to note that while SP cells are highly enriched for repopulating cells, at least 250 SP cells are required to achieve significant repopulation in mice [Goodell *et al.*, *J.Exp.Med.* **183**:1797-1806 (1996)] indicating that most SP cells are not true stem cells. In contrast, it has previously been shown that CD34-, c-kit+, Sca1+, lineage negative (CD34-KSL) cells from the bone marrow are a relatively pure subset of repopulating cells, with repopulation in about 20% of mice that are transplanted with single sorted cells [Osawa *et al.*, *Science* **273**:242-245 (1996)]. Therefore, transporter expression was studied in the highly purified CD34-KSL population, as well as from a number of other different sorted populations. Like SP cells, the CD34-KSL cells expressed relatively high levels of *Bcrp1* mRNA, however in contrast to SP cells, there were little to no expression of the other ABC transporters (Fig. 19). In the more differentiated CD34+ KSL cell fraction, there was marked downregulation of *Bcrp1* expression with the appearance of significant expression of *mrp1, 2,* and *4*. The S+K+Lin- population is a mixture of CD34- and + cells, and gave results that were intermediate between the CD34+ and - subfractions. *Bcrp1* expression was not detectable in granulocytes, macrophages, B cells, or thymocytes. The only other cell populations with detectable *Bcrp1* expression were erythroid progenitors (Ter119+) and natural killer cells (NK1.1+). These results suggest that *Bcrp1* expression is highly specific for repopulating stem cells in the lineage negative compartment of the

bone marrow, and that expression of other transporters in the sorted SP cell population were likely due to the presence of more differentiated cells with lesser degrees of repopulation potential. These data indicate that *Bcrp1* and/or BCRP expression should be useful markers for stem cell identification and purification. The expression data are also consistent with a necessary functional role for *Bcrp1* gene expression in repopulating hematopoietic stem cells, and perhaps in SP stem cells from muscle and other tissues.

Functional studies with a BCRP retroviral vector confirm dye efflux activity consistent with the SP cell phenotype: To gain further information regarding BCRP as a functional determinant of SP stem cells, a retroviral vector based on the Murine stem cell virus (MSCV) was constructed that expressed both the human *Bcrp* cDNA and a linked GFP marker gene. Ecotropic producer cells were generated and shown to express the BCRP protein by Western blot, using an polyclonal rabbit antibody that recognizes an internal epitope of BCRP. It was next determined whether BCRP could efflux Hoechst dye, a prerequisite for conferring the SP phenotype. Efflux studies done in the murine fibroblast producer cells clearly demonstrated that BCRP efficiently effluxed Hoechst dye, while neither the parental cells nor an MRP1 producer cell line showed any efflux activity (Fig. 20). The blue wavelength used for emission analysis did not overlap with the GFP emission wavelength, as was demonstrated using a producer cell line that coexpressed GFP and a DNA repair enzyme (MSCV-MGMT-GFP).

To further clarify the link between BCRP expression and the SP cell phenotype, the BCRP vector was used to transduce murine bone marrow cells. The transduced cells were grown in expansion cultures for 7 days and analyzed by FACS for expansion of the SP population. As a negative control, cells were transduced with the MSCV-MGMT-GFP vector and expanded in parallel. Whereas, a large expansion of phenotypic SP cells was seen with the BCRP vector, in marked contrast, there was a relative loss of SP cells in the control population (Fig. 21A). After 7 days of culture, about 22% of the population transduced with the BCRP vector fell within the SP gate (Fig. 21B), which constituted a significantly greater degree of expansion than was previously noted with the HaMDR1 vector (Fig. 8). The data from the BCRP

retroviral experiments further strengthen the association between BCRP expression and the SP phenotype of stem cells.

High levels of expression of BCRP mRNA in sorted SP cells from Rhesus Monkey bone marrow: To determine if primate SP cells were expressing BCRP, a bone marrow aspirate sample was obtained from a normal Rhesus Monkey. After lysis of the red blood cells, the leukocyte population was stained with Hoechst dye and analyzed by flow cytometry for SP cells. The flow pattern was very similar to that obtained with mouse bone marrow, with about 0.05% of cells falling into the SP gate (*see* above). Sorting was performed and resulted in isolation of 2000 SP cells, and 10,000 cells from a distinct gate outside of the SP region (non-SP cells). RNA was extracted, and a RT-PCR cycle curve using β-actin primers as an internal control showed roughly equivalent signals at 35 cycles for non-SP cells versus 60 cycles for SP cells (Figs.22A-22C). These PCR conditions were repeated using BCRP-specific primers in place of the β-actin primers. A strong signal was obtained with BCRP primers at 60 cycles in the SP cells sample (Fig. 22B), and a much fainter signal was detected at 50 cycles. No BCRP signal was detected at 35 cycles in the non-SP sample, which was the highest cycle number used for this sample (Fig. 22C). These results demonstrate relatively specific, high level expression of BCRP mRNA in monkey SP cells since the β-actin signal for non-SP cells at 35 cycles was actually greater than the signal for SP cells at 60 cycles. Taken together with the mouse data, (above) these results show that the expression of a BCRP transporter ortholog is conserved in SP stem cells from diverse species. In addition, these results further confirm that human stem cells can be identified and/or purified by monitoring/exploiting their unique BCRP expression .

Expression of Bcrp1 in murine myoblast SP cells: Stem cells bearing the SP phenotype have also been identified in murine muscle, and appear to be related to the satellite cells that are located on the periphery of the muscle fiber. Consistently, these cells also appear to be associated with muscle regeneration.

SP cells were therefore isolated from the murine muscle, and assayed for *Bcrp1* expression by RT-PCR to further correlate *Bcrp1* (BCRP) expression with the SP phenotype. Muscle tissue was dissected, minced, digested with collagenase, and a

single cell suspension was stained with Hoechst dye for SP cell analysis. An SP population of cells was observed with FACS analysis that bears a striking resemblance to the profile seen with bone marrow cells (Figure 22D). Gated myoblast SP cells were sorted, and RNA was prepared from a fraction of 20,000 cells. RT-PCR analysis showed relatively high levels of *Bcrp1* expression (Fig. 22E). However, unlike the results with monkey bone marrow, a distinct non-SP cell fraction was not available for analysis. These results further support the conclusion that *Bcrp1* expression can be used to identify SP stem cells from a variety of organs.

BCRP expression in blasts from pediatric acute myelogenous leukemia (AML). To determine if BCRP expression could be detected in leukemia cells from pediatric patients with AML, RT-PCR analysis was performed on RNAs derived from leukemic bone marrow from four individual cases (Figs. 23A-23B). Two cases were strongly positive for BCRP mRNA, with BCRP-amplified fragments detected at 25 cycles of amplification. Interestingly both of these "high-expressing" cases were associated with the M1 FAB phenotype. Two other cases with an M5 phenotype did not show any detectable signal at 25 cycles, but BCRP could be detected at 30 cycles. β-actin controls demonstrated that this variation was not due to differences in mRNA loading. The low level signal seen in the M5 cases could have been due to contaminating erythroid progenitors which may be expressing BCRP, or due to low level BCRP expression in the blast cells. These data confirm that BCRP mRNA expression can be detected in at least some primary AML samples. Furthermore, these results provide further impetus for using an anti-BCRP antibody to probe blast cell samples in the diagnosis/prognosis of AML.

The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. These documents, and all others cited above, should be considered as incorporated by reference in their entirety.

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While the invention has been described and illustrated herein by references to the specific embodiments, various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications in addition to the immediately foregoing are cited herein, the disclosures of which are also incorporated by reference in their entireties.